# POST-FIRE COLONIZATION OF *CISTUS CRETICUS* L. SEEDLINGS BY ECTOMYCORRHIZAL FUNGI IN ALEPPO PINE FORESTS IN CENTRAL GREECE.

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## Abstract

In mature forests, ectomycorrhizal fungal mycelia tend to be concentrated in the litter and upper soil layers and are therefore prone to damage by fires. Sources of fungal inoculum required by ectomycorrhizal tree and shrub seedlings re-establishing in burned forest sites may thus be reduced and patchily distributed.

*Cistus creticus* L is an ectomycorrhizal woody perennial that establishes after fires in Aleppo pine (*Pinus halepensis* Mill.) forests from small, hard-coated seeds that are stored in the soil. Field observations, and field and greenhouse bioassays were used to assess spatial and temporal variation in post-fire colonization of *Cistus creticus* seedlings by ectomycorrhizal fungi and to investigate the potential of resprouting shrubs to act as refugia of ectomycorrhizal fungal inoculum.

Six months after fire, there was spatial stratification of ectomycorrhizal fungal inoculum that was strongly related to depth in the soil profile and weakly to proximity to resprouting shrubs. Results show that the earliest post-fire colonization of *Cistus* seedlings throughout the forest is strongly dominated by E-strain fungi and an unidentified fungus (Unknown 1) that form weak ectomycorrhizas predominantly in the upper few centimetres of root systems. Early colonization of long roots in addition to short roots appears to be an important process in Unknown 1 which is replaced on short roots by a wide range of mature forest fungi by the sixth month after host seed germination. E-strain fungi, on the other hand appear able to persist, at least for the first six months of seedling establishment.

Results of a greenhouse bioassay suggest that the weak ectomycorrhiza-forming fungi colonize from spores or other resistant propagules while the mature forest fungi colonize from mycelia that are attached to an existing resource-base that may be either living or dying roots of mature forest plants. These strategies for fire survival are somewhat analagous to the 'seeding' and 'resprouting' of the aboveground vegetation.

Proximity to resprouting shrubs had little quantifiable effect on percentage colonization or ectomycorrhizal community structure associated with *Cistus creticus* seedlings though there was some circumstantial evidence to suggest that some mature forest fungi, particularly basidiomycetes, are restricted to soils around both ectomycorrhizal and non-ectomycorrhizal resprouting shrubs in the early stages of post-fire recovery. New roots of ectomycorrhizal resprouting shrubs such as *Quercus coccifera* are likely to be locally important sources of carbohydrate for ectomycorrhizal fungi recovering after fires. However, it is suggested that

by establishing in large numbers after fires and exhibiting broad receptivity to ectomycorrhizal associates, *Cistus* seedlings facilitate the post-fire recovery of mature forest fungi.

Early colonization of *Cistus* by weak ectomycorrhiza-forming fungi may provide quick access to the external sources of nutrition required by this small-seeded species yet at a low carbon cost to the establishing seedlings. Thus early interaction between plant seeders and their mycological counterparts facilitates the establishment of the *Cistus* seedlings that in turn facilitate the recovery of mature forest fungi.

As with the aboveground vegetation, the ectomycorrhizal fungal community in Mediterranean pine forests appears to be resilient to the effects of fire under natural conditions. However, perturbations to the fire regime could act to break the cycle of interaction that has evolved to confer that resilience.

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# Chapter 1 – General introduction

"Every day, somewhere in Europe there is a fire in a forest" (EU, 1996). It has been documented that during the years 1989-93, 2,600,000 ha of forest were burned in 225,000 separate forest fires across 224 provinces or departments in Greece, Spain, Italy, France and Portugal (EU, 1996). Management of the forest resource in the face of such dramatic disturbance requires a sound understanding of the ecological impacts of fire on forest ecosystems. Ecological research in this area has previously focused on the aboveground component of forests. Much less is known about the impact of fire belowground and yet the soil and its biota are integral to the functioning of forest systems. This thesis aims to address one aspect of this large gap in our knowledge by examining the impact of forest fires on ectomycorrhizal fungal communities and the dynamics of post-fire colonization of establishing seedlings.

## 1.1 Fire in the Mediterranean Basin

Fire is evidently an important feature of the ecosystems of the Mediterranean basin and has profound implications for both the human economy and the natural history of the region. In combination with an arid summer climate and poor soils, fire has contributed to the creation of many of the distinctive Mediterranean habitats we recognise today such as maquis, garrigue and phrygana (Tomaselli, 1977, 1981; Trabaud, 1981). While fire has probably been a natural agent working upon these habitats for many thousands of years, it is the history of human intervention and fire use that has had the most significant impact in shaping the landscape (Pyne, 1997).

Understanding the processes by which fire affects vegetation in Mediterranean ecosystems has received much attention from ecologists (Arianoutsou-Faraggitaki & Margaris, 1981, 1982; Naveh, 1975; Trabaud, 1990; Trabaud *et al.*, 1985a; Trabaud *et al.*, 1985b). Most of this has focussed on the aboveground processes involved in post-fire vegetation regeneration and this has yielded much useful information regarding individual plant strategies for survival. Not surprisingly, in common with other fire-prone regions of the world, plants in Mediterranean ecosystems are fire-adapted. Plants typically adopt one of several different strategies for surviving fires. In general these may be classified as fire resistance, resprouting and seeding. Individuals of fire resistant species survive due to the insulating properties of a thick bark as in the case of *Quercus suber*. Individuals of resprouting species survive as underground organs that produce new stems from underground buds after the fire.

Individuals of seeding species may be killed by the fire but regeneration of new individuals from canopy or soil stored seed is stimulated.

To date, relatively little work has been directed towards understanding the role of belowground processes in the dynamics of vegetation in Mediterranean-type ecosystems in the post-fire environment. This has been recognised as an area of research of outstanding importance (ModMED 2001).

Mycorrhizas have long been recognised as immensely important to the healthy growth of plants by enhancing nutrient and moisture acquisition potential and by offering resistance to soil-borne plant pathogens (Smith & Read, 1997). In Mediterranean soils, mycorrhizas may be of particular importance due to general nutrient deficiency and periods of water stress (Giannakis & Arianoutsou, 1997). Beyond the level of individual plants, it is becoming increasingly clear that mycorrhizas are important in ecosystem functioning through the formation of mycelial networks that help to regulate the pathways of nutrient exchange (Helgason *et al.*, 1998; Rayner, 1998; Sen, 2000; Setala *et al.*, 1999)

Understanding the ways in which forests function in nature is important if we seek to manage them for aesthetic and utilitarian purposes. Our understanding of them needs to be greater still if we are to attempt to restore forests to degraded areas. The need for restoration of Mediterranean pine forests is becoming more acute. Increased urban encroachment on the remaining forest areas has lead to an increase in the number of accidental and deliberate fires. The resulting increase in fire frequency and therefore reduced fire return-time means that increasing areas of forest are being subjected to second burns before regenerating trees have reached reproductive maturity. Once this happens, there is no further possibility of natural regeneration and there is then a requirement for afforestation if tree cover is to be restored. Success of afforestation programmes may be enhanced by greater understanding of natural forest processes, particularly those occurring belowground.

To date the impact of fire on mycorrhizal fungi in the Mediterranean Basin and the dynamics of early post-fire colonization of seedlings have received little attention and there is a need for primary research in this area. The current project was undertaken in order to address this research need in the context of Aleppo pine (*Pinus halepensis*) forests in Greece.

## 1.2 Aleppo pine forests of the Mediterranean basin

### 1.2.1 Distribution

The Aleppo pine, *Pinus halepensis,* is the most widely distributed of the Mediterranean pines occurring from the Iberian peninsular in the west to the eastern limit of the Mediterranean Basin in Israel and Lebanon. It is unique among the Mediterranean pines in having a considerable part of its natural range occurring on the southern fringes of the Basin in North Africa (Barbero *et al.*, 1998). Being extremely tolerant of dry conditions, *P. halepensis* thrives in the thermo-Mediterranean *etage* of these regions and dominates in stable plant communities or in transitional communities that are later dominated by broad-leaved evergreens such as *Quercus ilex.* Progressive abandonment of marginal agricultural land over the last 100 years has facilitated extensive natural regeneration of the Aleppo pine on formerly cultivated ground (Quézel, 2000).

The success of the Aleppo pine in the Mediterranean Basin is largely a function of its broad ecological tolerance. This species is tolerant of low nutrient availability and in general the favoured substrate is basic, principally marl, limestone and dolomite (Barbero *et al.*, 1998). Rainfall varies over its entire distribution range between 200 and 1500 mm per annum though it is found most abundantly within semi-arid to sub-humid zones (400 – 800 mm per annum) (Quézel, 2000). In the southern Mediterranean lands it can occur from sea level up to 1400 m with isolated patches and individuals occurring as high as 2800 m in the Central High Atlas (Quézel, 2000). In the northern Mediterranean countries however, it tends to occur from sea level up to 800 m.

In Greece, the Mediterranean pines *Pinus halepensis* and *P. brutia* together cover an estimated 8.7% of the forested area and are characteristic of both limestone and sandy soils in coastal regions (Kazanis & Arianoutsou, 1996). *Pinus halepensis* occurs up to 600-800 m and is particularly abundant in the Peloponnese, Attica, Euboea, western Halkidiki and many of the Ionian and Aegean Islands (Quézel, 2000; Tsitsoni, 1997).

The understorey of mature Aleppo pine forest in Greece is dominated by sclerophyllous shrubs such as *Quercus coccifera, Pistacia lentiscus, Phillyrea latifolia, Olea europaea, Arbutus unedo* and *A. andrachne* while dwarf-shrubs of the *Cistaceae* such as *Cistus* and *Fumana* spp. are also common (Kazanis & Arianoutsou, 1996).

These species represent a mixture of mycorrhizal types. Species in the Cistaceae are ectomycorrhizal and the relevant literature on these associations is reviewed in Chapter 2. Of the other dominant species *Quercus coccifera* is also ectomycorrhizal while *Pistacia lentiscus, Phillyrea latifolia* and *Olea europaea* are considered arbuscular mycorrhizal (Puppi & Tartaglini, 1991). *Arbutus unedo* belongs to a small group of plant species within the Ericaceae which form arbutoid mycorrhizas. These are similar to ectomycorrhizas in the

development of a Hartig net and mantle but are characterised by extensive intracellular penetration of hyphae in the first layer of cortical cells (Smith & Read, 1997). It is now generally accepted that arbutoid mycorrhizas are formed by species of fungi that form ectomycorrhizal associations with other hosts (Danielson, 1984b; Molina & Trappe, 1982; Zak, 1974, 1976a, b). At present the only confirmed fungal symbiont in a Mediterranean species of *Arbutus* (*A. unedo*) is *Cenococcum geophilum* which has been observed on field-collected root samples (Giovannetti & Lioi, 1990).

### 1.2.2 Vegetation response to fire

In common with other fire-prone ecosystems, post-fire regeneration of vegetation in Mediterranean pine forests comes about by the resprouting of surviving underground storage organs of understorey shrubs or by the release of seed from canopy or soil seed banks (Arianoutsou, 1998; Arianoutsou & Ne'eman, 2000; Daskalakou & Thanos, 1996). Among the woody species of the forest, the sclerophyllous understorey shrubs are generally resprouters while *Pinus halepensis* and the dwarf shrubs in the Cistaceae are the principal seeders.

The heat of summer wildfires induces opening of the serotinous cones of *P. halepensis* and release of their seeds (Nathan & Ne'eman, 2000). Seeds germinate on the forest floor with the coming of the first autumn rains that generally arrive in October. These same rains also stimulate the germination of soil seed banks that are dominated by members of the Leguminosae and Cistaceae (Kazanis & Arianoutsou, 1996; Papavassiliou & Arianoutsou, 1993).

*Cistus* is a very common fire-following genus in the Cistaceae in the Mediterranean Basin. It has small, hard-coated seeds that lie dormant in large numbers in the pre-fire soil and are stimulated to germinate once the moisture of the first rains penetrates their fire-scarified coats (Thanos & Georghiou, 1988; Thanos *et al.*, 1992). This results in a flush of seedlings growing around and between the resprouting stems of the understorey shrubs. Seedling mortality tends to be high during the first summer after the fire but thereafter populations remain stable (Arianoutsou & Ne'eman, 2000). Individuals grow quickly and can attain reproductive maturity within one to two years but they appear to be short-lived and die after 12-15 years (Arianoutsou, 1998; Roy & Sonié, 1992).

*Cistus creticus* was chosen as a model species for the investigation into post-fire mycorrhizal dynamics because it is known to be ectomycorrhizal and is an extremely common and

important component of the regenerating vegetation. It is also very easy to propagate which facilitates greenhouse experimentation.

## 1.3 Ectomycorrhizas in forest ecosystems

### 1.3.1 Ectomycorrhizas

While there are several types of mycorrhizal association that may be important in forest functioning, this thesis is concerned principally with ectomycorrhizas (EM). EM have traditionally been defined as consisting of three components: Hartig net, mantle and extra-radical hyphae (Smith & Read, 1997). Hartig net is the name given to the labyrinthine network of branched hyphae that surround root epidermal and cortical cells. The mantle is the sheath of fungal hyphae that forms over the root surface. Extra-radical hyphae are those that extend away from the root and mantle into the surrounding soil. With EM, hyphae inside the root never penetrate the root cells. This differentiates them from ectendomycorrhizas in which the same structural features may be present but in which hyphae penetrate root cells to form intracellular haustoria.

There is now a vast literature on the physiological aspects of ectomycorrhizal symbiosis. Rather less is known about the ecology of this important association in nature. Here I will give a brief overview of the aspects of ectomycorrhizal ecological research that are relevant to the present investigation of the effects of fire on ectomycorrhizal communities and how this impinges on post-fire recovery of both plants and fungi.

### 1.3.2 Symbionts, specificity and fungal networks

Current estimates suggest that some 5000-6000 species of fungi in the Ascomycotina and Basidiomycotina form ectomycorrhizas (Molina *et al.*, 1992). Ectomycorrhizas appear to be largely restricted to woody perennials but within this grouping there is diversity among ectomycorrhizal fungi in the degree of specificity to hosts. The range encompasses fungi restricted to a single genus, family or phylum (angiosperms or gymnosperms) or with little to no host restriction (Molina *et al.*, 1992).

Where fungi exhibit low levels of specificity, the possibility of mycelial connections between plants of different taxa arises and this has given rise to the concept of fungal networks in forests, sometimes referred to as the "wood-wide web" (Sen, 2000). Fungal networks have the potential to influence several important ecosystem processes including

nutrient recycling within plant individuals, nutrient exchange between mature plant individuals and facilitation of seedling establishment.

Networks of fungal hyphae may play an important role in recycling nutrients lost from plant individuals. Hyphae of EM fungi attached to the roots of a plant may play a role in scavenging for the products of decomposition of litter and returning them directly to the plant. The products of decomposition include nutrients in free inorganic form and also bound up in organic forms such as amino acids. Culture studies indicate that some EM fungi can utilize nutrients from such organic sources (Tibbett *et al.*, 1998; Yamanaka, 1999). Furthermore, experimental studies conducted with natural substrates suggest that some EM fungi can produce the degradative enzymes required to mobilize nutrients directly from complex organic sources (Bending & Read, 1995a, b) and return them to their host plant (Perez-Moreno & Read, 2000). Facilitation of such tight nutrient recycling may be particularly advantageous in situations where EM plants grow in isolation in nutrient poor soils such as the savannah woodlands of Africa and some forms of semi-arid shrublands such as garrigue in the Mediterranean Basin.

Molecular studies of sporocarps have revealed that individual clones of some ectomycorrhizal fungi can be 20-30 m in diameter (Anderson *et al.*, 1998; Dahlberg & Stenlid, 1995) and even up to 100 m across (Anderson *et al.*, 1998; Dahlberg & Stenlid, 1995; Fiore-Donno & Martin, 2001). Although fragmentation of genets may result from fungivore grazing and soil disturbance, this indicates a potential for mycelia of EM fungi to extend well beyond the root systems of individual plants. Where mycelial networks extend beyond the canopy zone of one individual and colonize others they may facilitate the exchange of carbon and nutrients between plants of the same and different taxa (Newman, 1988; Perry *et al.*, 1989). This may be of particular importance to the establishment of seedlings in situations where either light or nutrients or both are limiting. Seedlings that can become connected to the mycelial networks associated with their 'mother' tree or other mature individuals are quickly linked to an enhanced supply of minerals, water and carbon, transferred from adult to seedling *via* fungal hyphae.

Net transfer of carbon between different ectomycorrhizal tree species has been demonstrated in the field with the use of radiolabelled carbon isotopes (Simard *et al.*, 1997a). Trenching experiments have also been conducted in which tree seedlings were planted in soil that was effectively isolated from the fungal network associated with adjacent mature trees (Simard *et al.*, 1997b). Isolated seedlings had less of their root systems colonized by fewer ectomycorrhizal fungi than non-isolated seedlings and also had lower net photosynthetic rates and a smaller height:diameter ratio. Although it is possible that the trenching operations

altered the soil conditions detrimentally for the isolated seedlings the results of this experiment highlight an important aspect of forest mycorrhizal ecology, namely, the epidemiology of ectomycorrhizal fungi which is itself related to concepts of ectomycorrhizal succession.

### 1.3.3 Succession and epidemiology

The concept of a succession of ectomycorrhizal fungi colonizing newly establishing trees was originally based on observations of fruitbodies appearing beneath birch trees planted into a former agricultural field (Deacon *et al.*, 1983; Ford *et al.*, 1980; Last *et al.*, 1985; Mason *et al.*, 1982; Mason *et al.*, 1983). As such it was based on observations of the unfolding of an essentially primary succession in which a clear temporal separation between species was observed in the production of fruitbodies. Those that appeared first were termed 'early-stage' and those that appeared later, 'late-stage'. Furthermore, during the course of several years of recording, fruiting of the two groups of species became temporally concordant but spatially separated. The early-stage fungi, typically species of *Hebeloma*, *Inocybe* and *Laccaria*, tended to produce fruitbodies at the peripheries of the tree root zone where new roots were being extended into unexploited soil. Late-stage fungi, typically species of *Lactarius, Cortinarius,* and *Russula* tended to appear from the older parts of the root systems close to the tree trunk (Mason *et al.*, 1982). An analysis of *in situ* ectomycorrhizal root tips showed the same pattern of spatial separation with respect to distance from the trunk (Deacon *et al.*, 1983).

Subsequent work on the relevant fungi has shown that late-stage and early-stage fungi appear to differ in several important functional attributes and adopt different regenerational strategies that help to explain their position in the succession (Deacon *et al.*, 1983; Fleming, 1983, 1984, 1985). Among the key characteristics of the early-stage fungi is the ability to form mycorrhizas in unsterile soils from spore and mycelial fragment inocula. Their spores germinate readily *in vitro* and they have a low sugar demand for growth in culture (Deacon & Fleming, 1992). Late-stage fungi on the other hand have recalcitrant spores *in vitro* and a high sugar demand and do not readily colonize new roots in unsterile soils from spores or mycelial fragments. The results of coring and trenching experiments suggest that the primary sources of inoculum for new colonization events by late-stage fungi are the extra-matrical mycelium and rhizomorphs associated with intact, living mycorrhizas attached to mature hosts (Fleming, 1983, 1984; Simard *et al.*, 1997b).

It has been proposed that, at the forest stand scale, succession from early-stage to late-stage fungi is driven largely by changes in the quality and quantity of the resource base available

to the fungi as the stand matures (Last *et al.*, 1987). Early-stage fungi have a broad host range and are able to colonize disturbed or early successional habitats where litter is scarce and carbohydrate supplies from young plants are low. As the forest matures, the litter layer builds up and carbohydrate supply from host plants increases, favouring the greater proliferation of late-stage fungi that achieve dominance in the forest. This view of fungal succession has been questioned by Newton (1992) who argues that succession can be understood wholly in terms of the epidemiological characteristics of the fungi involved. The inoculum available to the first plants arriving at a new site is in the form of spores. It is the early-stage fungi that colonize readily from spores and so these appear first in the succession. As inoculum builds up, succession progresses via secondary infections from infected roots. Late-stage fungi are favoured in this process due to superior competitive abilities conferred, in many cases by the production of rhizomorphs that forage efficiently for new roots. However, not all late-stage fungi produce rhizomorphs and it seems likely that competitive ability in fungi is a combination of their resource base and their colonizing strategies, a view that was always implicit in the original proposal of the early-stage/late-stage concept.

Support for temporal succession of fungal fruitbodies can be found in studies that record observations of fruiting bodies in disturbed sites (SennIrlet & Bieri, 1999). However it is now well established that correspondence between production of fruitbodies and colonization of roots can be low (Gardes & Bruns, 1996; Gehring *et al.*, 1998; Jonsson *et al.*, 1999a; Jonsson *et al.*, 1999b; Yamada & Katsuya, 2001). Examination of EM communities below ground suggests that early-stage fungi can be common on roots in disturbed habitats and maintained at low frequency and abundance in mature forests (Mah *et al.*, 2001; Visser, 1995). This suggests that succession does not proceed as a replacement series, rather, as an additive one in which species richness increases with time but species evenness decreases. The increasing number of studies using molecular techniques that facilitate high resolution species discrimination have tended to reveal patterns of high dominance by a small number of species, typically members of the Russulaceae and Thelephoraceae, and a large number of species that are uncommon (Horton & Bruns, 2001), many of which are early-stage fungi.

Recent molecular studies looking at the size of ectomycorrhizal fungal genets in forests based on examination of sporocarp DNA have so far tended to support the view that early-stage fungi persist in mature forests *via* frequent colonization events from spore inocula. These studies show that early-stage fungi such as *Laccaria amethystina* and *Hebeloma cylindrosporum* tend to have large numbers of small genets indicating many independent mycelial establishment events from spores (Fiore-Donno & Martin, 2001; Gherbi *et al.*, 1999; Gryta *et al.*, 1997). In the latter species the aboveground pattern observed in

sporocarps corresponded with the belowground pattern observed on roots (Guidot *et al.*, 2001). The picture with late-stage fungi is less clear and in some cases appears contradictory to the traditional view of colonization by mycelial spread. Some studies show that late-stage fungi such as *Xerocomus* and *Suillus* species tend to have fewer, larger genets in a given area than early-stage fungi indicating higher levels of colonization by mycelial expansion (Dahlberg & Stenlid, 1995; Fiore-Donno & Martin, 2001). However, other studies have shown that late-stage fungi such as *Lactarius xanthogalactus* and *Russula cremoricolor* and *R. brevipes* can also exhibit patterns of high frequency of small genets indicating high levels of colonization from spores (Bergemann & Miller, 2002; Redecker *et al.*, 2001).

## 1.4 Effects of fire on ectomycorrhizal fungi in forest ecosystems

The magnitude of effect of fire on EM communities will be directly affected by the characteristics of the fire involved. Fire severity is a term that is used to describe the effect of a fire on various ecosystem components (Debano *et al.*, 1998). Fire severity may be influenced by fire intensity but is not the same thing. A high intensity crown fire can be of low severity at the soil interface if there is negligible transfer of heat downwards. Thus fire severity at the soil surface is a function of the nature and distribution of the fuel load in the forest.

Field observations suggest that EM fungal mycelia tend to proliferate in the litter and upper organic horizons (Buchholz & Gallagher, 1982; Buchholz & Motto, 1981; Griffiths *et al.*, 1991). It is in the litter layer and upper regions of the soil profile that the mycelial networks are both most abundant and most vulnerable to disturbance. Under conditions of low fire severity in forests with deep organic layers, ectomycorrhizal fungal communities may be little affected by fire (Jonsson *et al.*, 1999a). However, where fire occurs in forests with a well-developed understorey on shallow soils the effects may be more profound. In such situations, the immediate direct impact of a high severity fire is the removal or disruption of mycelial networks that are concentrated in the shallow litter and humus layers, reducing the colonizing potential of those fungi.

Reduction in abundance of EM fungi by as much as 90% after fire has been reported in Australian eucalypt and jarrah forests (Malajczuk & Hingston, 1981; Reddell & Malajczuk, 1984). An eight-fold decrease in total ectomycorrhizal biomass was recorded in burned Ponderosa pine stands compared to pre-burn values in the same plots (Stendell *et al.*, 1999). Active ectomycorrhizal root tips were lower in abundance in burned plots of mixed pine and oak in New Jersey, USA (Buchholz & Gallagher, 1982).

Measurements of decline in total ectomycorrhizal abundance are instructive but hide within them a greater complexity of response to fire at the individual species level. Differences may occur between fungal species in their distribution in the soil profile and their abilities to withstand and recover from the ravages of fire will be influenced by their dominant form of inoculum in the soil.

### 1.4.1 Post-fire recovery

As late-stage fungi may not initiate new colonization events very readily from spores or isolated mycelial fragments (Mason *et al.*, 1983), requiring instead to colonize new roots from established ectomycorrhizas that are connected to mature hosts (Fleming, 1983; Simard *et al.*, 1997b), recovery after disturbance is likely to be slow for these fungi. During the months immediately following disturbance, opportunistic fungi that can colonize new roots readily from spores may be able to take advantage of the resulting reduction in competition in the early post-fire stages.

Such a system of post-fire recovery that emphasises different colonization strategies has been proposed by Taylor & Bruns (1999) who differentiate between fungi of the mature forest community and the resistant propagule community. These terms are broadly analagous to 'late-stage' and 'early-stage' fungi respectively but are useful in getting away from the temporal connotations of the terms late-stage and early-stage that arose from the study of primary succession on young plantation trees in newly abandoned agricultural soils. In the context of secondary successions or in considering the dynamics of fungi in mature forests it is the ecological attributes of late-stage and early-stage fungi, that were also defined by the original studies, that are important.

To date, the most comprehensive study of post-fire recovery of EM fungi has been conducted in the Point Reyes National Seashore along the coast of California, USA. In October 1995, a wildfire burned approximately 5000 ha of coastal scrub and forest stands of bishop pine, *Pinus muricata*. Several studies that were designed to elucidate the ecology of EM fungi in undisturbed forest had been conducted in the same area before the fire. As it proved possible to exactly relocate some of the pre-fire sampling points, researchers were provided with a great opportunity to study the impact of the fire and fungal recovery.

Before the fire, the EM fungal community of the forest stand was dominated by a few species within the Russulaceae, Thelephoraceae and Amanitaceae with all other species being either rare or low in abundance (Gardes & Bruns, 1996). Pine seedlings establishing after the fire at an adjacent forest site were found to have formed mycorrhizas by the third

month after germination (Horton *et al.*, 1998). By the fifth month after germination, most of the same species present before the fire had formed mycorrhizas along with several other species, notably within the Ascomycotina. Thus genera such as *Amanita* and *Russula* which have been considered 'late-stage' fungi (Deacon *et al.*, 1983; Last *et al.*, 1983; Mason *et al.*, 1983), unable to colonise seedlings from spores or isolated mycelial inoculum under field condition, were found on five-month old seedlings in the burned sites. All of the trees in the study area were apparently killed in the fire and there were no ectomycorrhizal plants reported in the understorey. It appears that these fungi were able to colonize seedlings by mycelial transfer from dead roots of mature trees. This is supported by previous studies that have shown that detached mycorrhizal roots can remain infective in soils for up to eight months (Ferrier & Alexander, 1985; Harvey *et al.*, 1976).

In related studies, pre- and immediately post-fire greenhouse bioassays and field excavations suggested that resistant spores are the primary inoculum source for early post-fire EM formation by other fungi such as *Rhizopogon* spp., *Wilcoxina* sp. and *Tomentella sublilacina* (Baar *et al.*, 1999). Two *Rhizopogon* spp. were abundant on post-fire bioassay seedlings but absent or much reduced in abundance on field excavated seedlings from the same sampling locations (Baar *et al.*, 1999). They were also found to be much more common on seedlings growing in scrub sites compared to forest sites where the diversity of other EM fungi was greater (Horton *et al.*, 1998). These observations suggest that these *Rhizopogon* spp. maintain a store of resistant propagules in the soil analagous to soil seed banks in plants and that they colonize seedlings under conditions of reduced competition from fungi that colonize from vegetative mycelial sources.

One recurring feature of most fire studies is the prevalence of E-Strain fungi in post-fire ectomycorrhizal communities (Baar *et al.*, 1999; Grogan *et al.*, 2000a; Mah *et al.*, 2001; Miller *et al.*, 1998; Visser, 1995). Elsewhere, these fungi appear to be associated with disturbance in general (Laiho, 1965; Mah *et al.*, 2001; Mikola, 1965; Wilcox *et al.*, 1983). The success of E-Strain fungi in disturbed sites is in part due to the production of abundant resistant propagules in the form of thick-walled chlamydospores (Wilcox *et al.*, 1983). Fire-resistance of these propagules has been proposed to explain the association of E-Strain fungi with burned sites (Danielson, 1984b; Visser, 1995).

The only study that has addressed the impact of wildfire on the nature and availability of mycorrhizal inoculum in forests of the Mediterranean Basin indicated that E-Strain fungi are abundant in both burned and unburned soils (Torres & Honrubia, 1997). *Pinus halepensis* seedlings were grown in pots of forest soils for six months. At the end of that time, E-Strain fungi were completely dominant in all soil samples, occupying close to 100% of colonized

root tips (Torres & Honrubia, 1997). Only six other morphotypes were recorded at low abundance in the bioassay. Such high levels of colonization by E-Strain fungi appear to be unusual compared with their lower abundance in naturally establishing seedlings in other studies where they co-occur with greater numbers of other fungi. It may be that E-Strain fungi are sensitive to competition and become displaced from roots in the field by recovering fungi of the mature forest community. In the case of *Pinus halepensis* forests, it appears that few fungi are capable of colonizing from resistant propagules. If this is the case then the importance of E-Strain and other opportunistic post-fire colonizers may be most strongly felt in the very first months after seeds have germinated after the fire when competition from other fungi is at a minimum. Previous studies on post-fire EM community structure have tended to commence at least one year after the fires (Baar *et al.*, 1999; Danielson, 1984b; Grogan *et al.*, 2000a; Jonsson *et al.*, 1999a; Mah *et al.*, 2001; Miller *et al.*, 1998; Stendell *et al.*, 1999; Visser, 1995). There is a need to gain a wider understanding of colonization events in the early period after seed germination.

While EM fungi of the mature forest may be able to recolonize from dead roots after fire, studies suggest that their colonizing potential is much reduced when they are separated from living roots (Fleming, 1984; Simard *et al.*, 1997b). In situations where there are ectomycorrhizal plants that survive fires, production of roots by these may provide a vital refuge and resource base for EM fungi.

### 1.4.2 The potential role of refugia

That mycorrhizas may link plants of the same and different species has already been discussed. It has been suggested that such linkage may have a role in the regeneration strategies of both plants and fungi in disturbance-prone ecosystems. After disturbances, plant species that recover quickly by resprouting may act as refugia for the mycorrhizal fungi required by plant species that germinate from seed (Molina & Trappe, 1982; Perry *et al.*, 1992).

For example, in a study on outplanted Douglas fir seedlings on clear-cut and burned sites in south-western Oregon, those planted under the canopy of resprouting hardwood species were two times larger and had 60% more mycorrhizal root tips than those planted in the open (Borchers & Perry, 1990). They also found that seedlings grown under hardwood canopies and in the open differed in the pattern of dominance of mycorrhizal types. Similar findings have been reported for clear-cut forest plots in which seedlings planted next to living paper birch trees had greater EM diversity and abundance compare to seedlings planted outside the rooting zone of refuge trees (Kranabetter, 1999). Furthermore, in the latter study, the EM

community associated with seedlings planted close to the refuge trees was composed of the same fungi as in seedlings planted next to mature birch trees in undisturbed forest. Thus it appears that the refuge trees played a role in supporting communities of mature forest, late-stage fungi in disturbed areas.

In the case of Aleppo pine forests, ectomycorrhizal understorey shrubs such as *Quercus coccifera* may fulfil the role of refugia for fungi recovering after fires.

## 1.5 Seedling establishment after fires

### 1.5.1 The seed bed

The effect of fire on soil quality is determined largely by fire intensity at ground level but even in the hottest fires is confined to the upper few centimetres of the profile (Debano *et al.*, 1998). Chemical, physical and biological components of the soil are all affected by fire and effects can be beneficial or detrimental depending on the combination of site and fire characteristics.

At low to moderate intensities, short-lived increases in levels of available nitrogen and phosphorus that are beneficial to seedling establishment are well documented from a variety of ecosystems (Debano & Conrad, 1978; Ellis *et al.*, 1982; Grogan *et al.*, 2000b; Rashid, 1987; Romanya *et al.*, 1994; Trabaud, 1983) and Aleppo pine forests are no exception (Kutiel & Naveh, 1987; Kutiel & Shaviv, 1989). However, at higher fire intensities and in the longer term, burning can result in net export of nutrients from ecosystems *via* a combination of volatilization, and increased erosion (Gillon & Rapp, 1989; Rundel, 1983; Trabaud, 1994). Even at low intensity, fires can result in large losses of nitrogen due to the low temperature of volatilization of this element (Neary *et al.*, 1999).

Coupled with nutrient losses, alteration of soil structure through consumption of organic matter (Gillon *et al.*, 1999), particle aggregation (Giovannini *et al.*, 1990) and accumulation of hydrophobic compounds that reduce soil wettability (Debano, 1981) can lead to soil desiccation. Loss of structure also reduces soil stability and contributes to increased nutrient loss *via* hydrologic export. On steep slopes under conditions of high rainfall, nutrient-rich ash deposits can soon be lost or rearranged in mosaic patterns on the forest floor.

Heterogeneity of post-fire soil nutrients is further conferred by differences in ash quality between plants. Under experimental conditions Kutiel & Shaviv (1992) found that soil taken from Aleppo pine forests and burned with pine (*Pinus halepensis*) leaves and twigs

contained higher concentrations of ammonium and nitrate ions than soils burned with oak (*Quercus calliprinos*). Simulated leaching eliminated the differences between the soils and contributed to the conclusion that microbial inhibitors were at higher levels in oak ash. Interestingly, increases in ammonium and available phosphorus were also lower under a species of oak (*Quercus turbinella*) than under *Cercocarpus betuloides* in burned Chaparral though this was partly attributed to greater litter accumulation under the latter species prior to the fire (Overby & Perry, 1996).

Further heterogeneity in the quality of available nitrogen can arise from variation in ash deposition and consumption of organic matter which in turn is the result of within-site variation in fuel load and also, therefore, fire intensity. Inputs of basic cations released from soil organic matter and aboveground biomass generally lead to an increase in soil pH. Furthermore, the combination of organic matter combustion and ash deposition of minerals previously stored in above-ground biomass results in reduction of carbon-to-nitrogen and carbon-to-phosphorus ratios (Gillon *et al.*, 1999). This in turn stimulates microbial activity and increases mineralization rates and hence the ratio of nitrate to ammonium (Stewart *et al.*, 1993). Ions mobilised from bound organic sources also become vulnerable to loss *via* leaching.

It is apparent that the immediate post-fire soil environment is extremely heterogeneous in nutrient quantity and quality and subject to rapid leakage of nutrients away from the system. Together with desiccating conditions conferred by loss of soil structure, these factors can provide challenging conditions in which seedlings must establish.

### 1.5.2 Seedling establishment in a heterogeneous environment

Seedlings establishing in such a challenging environment require strategies for maximising their chance of having sufficient nutrients to ensure early growth and to maintain that growth in the face of declining levels of nutrients. Strategies that benefit seedlings at this stage may be adaptations that have arisen in response to more general deficiencies in soil nutrients that are common in fire-prone Mediterranean-type ecosystems. Several specialisms in nutritional mode have been recognised from nutrient-poor, fire-prone ecosystems from around the world. These include formation of mycorrhizas, fixation of atmospheric nitrogen *via* symbioses with nodule-dwelling bacteria, formation of cluster roots, carnivory and parasitism (Lamont, 1984).

For ectomycorrhizal plant species establishing after fires, the degree of fungal specificity may be important. It is apparent that after fires ectomycorrhizal inoculum may be patchily

distributed and relatively slow to recover. This may exert a selective pressure for low specificity for fungal partners among seedlings of ectomycorrhizal species establishing after fires. Low specificity would serve to maximise the probability of colonization. This may be particularly important in species that have small seeds. Allsopp & Stock (1992) have argued that small seed size in mycorrhizal plants is related to high levels of mycorrhizal dependency because of the early requirement for external sources of nutrition. Therefore, where fungal inoculum is patchy the ability to form associations with whatever fungi happen to be encountered would be advantageous.

Conversely, Janos (1980) has argued that large seed size is an advantage to obligately mycorrhizal species as large seeds act as an insurance against delayed or reduced mycorrhiza-formation where inoculum levels are low or infection is impeded. In the context of ectomycorrhizal associations, the apparent contradiction between the two theories is resolved if the question of host receptivity is considered. Obligately mycorrhizal species with narrow receptivity to partner fungi would benefit from having large seeds when establishing under conditions of reduced and heterogeneous inoculum such as during primary successions and after disturbances. Broad receptivity to fungi may enable obligately mycorrhizal species to derive the benefits attendant on production of smaller seeds.

## 1.6 Aims and objectives

The literature suggests that fires in pine forests have the potential to affect ectomycorrhizal fungal communities quite severely. Previous studies have tended to treat the post-fire forest floor as a homogeneous entity and quantify the impact of fire in terms of changes in species diversity with little attention paid to factors that might affect the spatial distribution of inoculum in soils. In particular the role of resprouting shrubs in promoting the recovery of fungal populations after fires requires more detailed examination. Given the pre-fire distribution of the bulk of ectomycorrhizal fungal mycelia in the litter and upper organic horizons and the known insulating properties of soil, an investigation of the depth distribution of inoculum in the upper layer of the soil profile may also be informative.

Recognition of the dynamic nature of community processes leads me to question the rather static view of post-fire ectomycorrhizal community structure that is presented by studies conducted at a single time, often many months after the fire. Fungi interact with one another and the abiotic environment and community composition one year after the fire may be quite different to that one month after the fire. Thus an assessment of the temporal development of the fungal community from the earliest stages after fire is required.

Knowledge of the broad epidemiological traits of ectomycorrhizal fungi that emerged from the early work on fungal successions can be used to investigate the dynamics of post-fire recovery. By contrasting the behaviour of different fungal species in greenhouse bioassays of forest soils and when colonizing naturally establishing seedlings, we can begin to elucidate the processes by which fungi recover after fires.

The aim of the project was to use *Cistus creticus* seedlings as bait plants to investigate these aspects of the spatial and temporal dynamics of early post-fire ectomycorrhizal fungal communities in Aleppo pine forests in Greece. In order to do this however, knowledge of the ectomycorrhizal morphotypes involved was required. *Cistus* has received attention from mycorrhizal researchers for its known association with species of forest truffles elsewhere in the Mediterranean Basin (see Chapter 2). However little work has been done to assess the extent of the ectomycorrhizal community associating with this genus in natural situations. Furthermore, very little mycorrhizal work at all has been carried out in Greece. Thus, one of the necessary goals of the present project was to describe the ectomycorrhizal associations of *Cistus creticus* seedlings in nature. This would provide an underpinning resource with which to address the following ecological questions.

1. Is there spatial patterning in the post-fire distribution of ectomycorrhizal fungal inoculum that is related to proximity to resprouting shrubs and/or depth in the soil profile?

2. After fires, do ectomycorrhizal fungi colonize seedlings primarily from vegetative mycelia attached to intact root systems or from resistant, isolated propagules such as spores or mycelial fragments?

3. How does the post-fire ectomycorrhizal fungal community compare with that of the unburned forest?

4. What are the dynamics of ectomycorrhizal colonization in the first few months after seedling germination?

Answers to these questions should improve our understanding of the ways in which fire affects this important component of the belowground environment.

# Chapter 2 – Mycorrhizal associations of *Cistus creticus* L.

## 2.1 Introduction

Species within the genus *Cistus* are well known to European mycorrhizal researchers as hosts to various species of forest truffle (*Tuber* spp.). Research has tended to focus on *in vitro* inoculation of *Cistus* plants with truffle inoculum and morphological and molecular characterisation of ectomycorrhizal root tips formed by these fungi. To date relatively little attention has been focused on the mycorrhizal associations of *Cistus* in nature.

The purpose of this chapter is to introduce the literature on mycorrhizal associations in the Cistaceae, to describe the methods used in the present study to investigate the mycorrhizal associations of *Cistus creticus* and to present and discuss the findings of those investigations.

## 2.2 Literature

The literature suggests that *Cistus* and other species in the Cistaceae form associations with a range of symbionts that include both ecto- and arbuscular mycorrhizal fungi.

### 2.2.1 Ectomycorrhizas

Using aseptic synthesis techniques *Cistus creticus* (syn. *C. incanus*) has been demonstrated to form ectomycorrhizas with five species of *Tuber* (*T. melanosporum* Vitt., *T. albidum* Pico, *T, brumale* Vitt., *T. aestivum* Vitt. and *T. rufum* Pico) (Fontana & Giovannetti, 1979; Giovannetti & Fontana, 1982). Five other species of *Cistus* (*C. albidus* L., *C. crispus* L., *C. laurifolius* L., *C. monspeliensis* L. and *C. salvifolius* L.) have been demonstrated to form ectomycorrhizas in synthesis with *Tuber melanosporum* (Giovannetti & Fontana, 1982).

The anatomy and ultrastructure of the association formed between *Cistus creticus (*syn. *C. incanus*) and *Tuber melanosporum* has been described (Fusconi, 1982, 1983). In this association initiation and development of the mantle proceeds from the root apical tip by growth of extramatrical hyphae into the dead layers of the root cap. The Hartig net extends beyond the root epidermal layer into the first layers of the cortex but does not reach the endodermis (Fusconi, 1982; Giovannetti & Fontana, 1982). This type of 'cortical Hartig net', which appears to be the norm for *Cistus* ectomycorrhizas (Giovannetti & Fontana, 1982), is more commonly seen among gymnosperms but has also been described in the angiosperm genera *Dryas* and *Populus* (Smith & Read, 1997).

Other ectomycorrhizal associations demonstrated within the genus *Cistus* are *Hebeloma saccharioliens* Quel. with *Cistus salvifolius* L. (Malloch & Thorn, 1985; Rosell, 1981) and *Laccaria laccata* (Scop.: Fr.) Berk. and Br. and *Boletus rhodoxanthus* Kallenb. with *Cistus ladanifer* L. (Hahn, 2001; Torres *et al.*, 1995).

Elsewhere within the Cistaceae, Read *et al.* (1977) reported that *Helianthemum chamaecistus* L. seedlings were colonised by brown septate hyphae growing on the root surface and between the outer cortical cells. These hyphae were attributed to *Cenococcum graniforme* (Sow) Ferd. and Winge which formed typical *Cenococcum* ectomycorrhizal root tips by the 2-4 true leaf stage. Short, black ectomycorrhizas typical of *Cenococcum* were also recorded on the roots of mature plants along with a second, unidentified morphotype. *Cenococcum* mycorrhizas were also found in samples of mature *Helianthemum canum* (L.) Baumg. and *H. appeninum* (L.) Miller.

Unspecified ectomycorrhizal associations were reported in three North American species of *Helianthemum* (*H. bicknellii* Fern., *H. canadense* (L.) Michx., *H. scoparium* Nutt. var. *scoparium*) (Malloch & Thorn, 1985). More recently, ectomycorrhizas formed by an unknown fungus with *Helianthemum ovatum* (Viv.) Dun. have been described in detail (Kovacs & Jakucs, 2001).

Associations between species of *Helianthemum* (*H. guttatum* Mill., *H. ledifolium* (L.) Mill. and *H. salicifolium* (L.) Mill.) and desert truffles in the genera *Terfezia*, *Tirmania* and *Phaeangium* have also been documented (Alsheikh & Trappe, 1983; Dexheimer *et al.*, 1985; Fortas & Chevalier, 1992; Morte *et al.*, 1994). There was much variablity in the structure of these associations ranging from endo- and ectendomycorrhizal to ectomycorrhizal. For example, (Dexheimer *et al.*, 1985) found that *Helianthemum salicifolium* inoculated with the desert truffle *Terfezia claveryi* Chat. (Pezizales) formed an endomycorrhizal association in which hyphae penetrated the root cortical cells but did not form a Hartig net or mantle. *Terfezia leptoderma* Tul. inoculated onto the same host species formed a weak ectomycorrhizal association in which intracellular penetration and a mantle were absent but a Hartig net extending as far as the vascular cylinder was formed (Dexheimer *et al.*, 1985). A later study showed that the structure of the mycorrhizal association formed *in vitro* between *Helianthemum guttatum* and *Terfezia claveryi* as well as two other desert truffles (*Terfezia arenaria* (Moris) and *Tirmania pinoyi* (Maire)) was influenced by phosphorus concentration (Fortas & Chevalier, 1992). Under conditions of high phosphorus all three truffle species formed weak ectomycorrhizas with fully developed Hartig net but no mantle. At low phosphorus concentrations ectendomycorrhizas were formed with Hartig net and intracellular haustoria but again, no mantle.

Another member of the Cistaceae, *Fumana procumbens* (Dun.) Gr. Godr., has been demonstrated to form ectomycorrhizas with *Tuber aestivum*, *T. brumale* and *T. melanosporum* (Chevalier *et al.*, 1975; Malloch & Thorn, 1985) as well as *Hebeloma ammophilum* Bohus (Jakucs *et al.*, 1999) and *Inocybe heimii* Bon (Magyar *et al.*, 1999).

Unspecified ectomycorrhizal associations have also been reported in *Hudsonia ericoides* L., *H. tomentosa* Nutt., *Lechia intermedia* Legg., *L. villosa* Ell. (Cistaceae) (Malloch & Thorn, 1985).

### 2.2.2 Arbuscular mycorrhizas

Arbuscular mycorrhizas (AM) are generally found in the roots of a very wide range of herbaceous plants (Smith & Read, 1997). However, some woody perennials within several families including Myrtaceae (*Eucalyptus*), Salicaceae (*Populus*, *Salix*), Betulaceae (*Alnus*), Caesalpinaceae (*Afzelia*) and Uapacaceae (*Uapaca*) are known to form both EM and AM associations (Molina *et al.*, 1992; Moyersoen & Fitter, 1999). More recently, several gymnosperms including species of *Pinus, Abies* and *Tsuga* which have traditionally been considered as strongly ectomycorrhizal, have been reported as forming arbuscular mycorrhizas at the seedling stage (Cazares & Trappe, 1993; Horton *et al.*, 1998).

*Helianthemum* in the Cistaceae is included among the genera listed by Molina *et al.* (1992) as forming both types of mycorrhiza. Young field-excavated seedlings of *Helianthemum chamaecistus*, ranging in developmental stage from cotyledons alone to four true leaves, have been reported to "... commonly show vesicular-arbuscular infection." as well as formation of typical EM though the nature of the AM structures observed was not detailed (Read *et al.*, 1977). Furthermore, in addition to the presence of ectomycorrhizal roots reported by Malloch & Thorn (1985) in *Helianthemum bicknellii*, *H. canadense*, *H. scoparium* var. scoparium, *Hudsonia ericoides*, *H. tomentosa*, *Lechia intermedia* and *L. villosa*, the authors noted that "...many of the roots lacked mycorrhizae altogether or had vesicular-arbuscular mycorrhizae." However no further information on the structures seen was offered. Colonization by both EM and AM fungi has also been reported in *Cistus monspeliensis* (Puppi & Tartaglini, 1991). However this was based on a single observation of glomalean-like vesicles in roots (Puppi, pers. comm.).

Given these observations, the status of *Cistus* as a potentially dual-mycorrhizal genus evidently requires clarification.

### 2.2.3 Post-fire ascomycetes

In addition to the traditional groups of mycorrhizal associations, another group of fungi that may interact with plants in the present context of establishment in burned forests is the post-fire ascomycetes. Although no such interactions have previously been investigated for plants of the *Cistaceae* they have been reported for *Pinus, Eucalyptus* and *Melaleuca* and these are reviewed here as a precursor to a synthesis experiment between *Cistus creticus* and three species of post-fire ascomycetes.

Colonization of burned ground by numerous species of ascomycetes within the order Pezizales and a few species within the Agaricales is well known (Carpenter & Trappe, 1985; Petersen, 1970; Wicklow, 1988; Zak & Wicklow, 1980). Interactions between some of these fungi and the roots of *Pinus contorta* in pure culture synthesis have been investigated. Egger & Paden (1986a, 1986b) found that *Anthracobia maurilabra* (Cooke) Boud., *A. tristis* (Bomm., Rouss. & Sacc.) Boud., *Tricharina praecox* (Karst.) Dennis var. *intermedia* Egger, Yang & Korf, *Geopyxis carbonaria* (Alb. & Schwein.) Sacc., and *Trichophaea hemispherioides* (Mont. ) Graddon all interacted to some extent with the roots of *Pinus contorta* in pure culture synthesis. Interaction was generally indicated by varying amounts of mantle formation that was often highly localised. Some penetration into the cortical cell layers with a rudimentary Hartig net formed in some cases. These fungi were generally regarded as pathogenic to a greater or lesser extent on the basis of host reactions (secondary metabolites, lignification, cell disruption, chlorosis, reduced radicle extension) though the possibility of some species being facultatively mycorrhizal was recognised. This view has been supported by the recent demonstration that *Geopyxis carbonaria* (Alb. & Schwein.) Sacc. is a biotrophic root associate with *Picea abies* in Norwegian forests, forming brown, smooth, mycorrhiza-like root tips (Vrålstad *et al.*, 1998).

Other species of post-fire ascomycetes in the Pezizales have been demonstrated to form true ectomycorrhizas in pure culture synthesis (Warcup, 1990). *Boudiera tracheia* (Rehm ex Gamundi) Dissing & Schumacher, *Labyrinthomyces varius* (Rodway) Trappe, *Lachnea vinosobrunnea* (Berk. & Br.) Sacc., *Muciturbo reticulatus* Talbot, *Peziza whitei* (Gilkey) Trappe, *Plicaria alveolata* ((Rodway) Rifai, *Nothojafnea cryptotricha* (Rifai), and *Pulvinula tetraspora* (Hansford) Rifai, were isolated from ascocarps and synthesised with *Eucalyptus obliqua* L. Hér. or *Melaleuca uncinata* R. Br. ex Aiton f.. All associations were reported to produce marked growth responses compared with uninoculated plants though the nature of the response was not elaborated.

These reports suggest that interactions between strongly fire-following species like *Cistus creticus* and species of post-fire ascomycetes are worth investigating.

## 2.3 Aims

This chapter summarises the mycorrhizal status of *Cistus creticus* seedlings during the early establishment phase in burned *Pinus halepensis* forests. First, an outline will be given of the methods used throughout this study to differentiate and to putatively identify some of the fungal taxa involved in the formation of ectomycorrhizas will be given. This will be followed by details of the use of a random access computer key to store morphotype descriptions in a readily accessible form. Methods used for clearing and staining roots for the observation of arbuscular and other endomycorrhizal fungal structures using light microscopy are also described. This is followed by a description of a synthesis experiment in which three isolates of post-fire ascomycete fungi were inoculated onto axenic *Cistus creticus* seedlings.

A description of the mycorrhizal status of this species will be presented including an inventory of ectomycorrhizal morphotypes that will largely refer to an Appendix of morphotype descriptions and photographs. Finally I will present a brief discussion of some of the issues involved in considering the use of traditional and modern techniques and a plea for the adoption of standardized terminology in the description of EM morphotypes.

## 2.4 Methods

### 2.4.1 Morphotyping

Ectomycorrhizal root tips were described according to the general methods set out in the literature (Agerer, 1987-2001; Goodman *et al.*, 1996-2000; Ingleby *et al.*, 1990). The terminology adopted for describing ectomycorrhizas was that of Agerer (1987-2001).

Using these methods, descriptions were made at two levels: macroscopic and microscopic. At the macroscopic level, morphological features of ectomycorrhizas were made using a dissecting microscope at x6 to x50 magnification. Features described included the shape and colour of colonized root tips, surface texture, lustre and mantle integrity. The form and frequency of emanating elements such as cystidia, hyphae and rhizomorphs were also described.

Moving to the microscopic level, colonized root tips were mounted in Cotton blue or Toluidine blue and described with the aid of a compound light microscope at magnifications of x100 to x1000. Differential Interference Contrast (DIC) was regularly employed to highlight particular features. At the higher magnifications, hyphae and surface elements were

described and measured. Where possible, mantle pattern was elucidated by peeling the fungal sheath from root tips and mounting these fragments in PVLG for examination at high magnification.

Voucher specimens of EM root tips were stored in 2% glutaraldehyde and several permanent reference slides exhibiting pertinent features were made for each morphotype.

### 2.4.2 Use of Random Access Key

As the number of morphotypes encountered grew, it became desirable to keep accessible records of morphotype descriptions against which new specimens could be easily compared. For this purpose, computer software, developed by Colin Legg of the University of Edinburgh, was used. This software comprises two applications: a database in which the characteristics of each morphotype are scored and a random access identification key that accesses the information stored in the database (Legg, 1992a, 1992b). Morphotype characteristics were scored on a scale ranging from one (very rare) to seven (always present). An example of the form used for scoring morphotypes and drawing relevant features is presented in Appendix 1. Once the database had been constructed, the random access key could be used to query it. For example, if the current specimen had tuberculate mycorrhizas with emanating rhizomorphs, the key program could be instructed to find all the morphotypes with these two characteristics in the database of previously described morphotypes against which to compare the current type.

One feature of the random access identification application allows the user to link to a web-page that has been constructed for each morphotype described in the database. These pages allow for the inclusion of lengthier descriptions, photographs illustrating the main features of each morphotype and additional information such as the location and description of collection sites.

### 2.4.3 Clearing and staining

For clearing and staining of roots to examine internal colonization, root systems were cut into fragments of approximately 1 cm length and placed in labelled modified syringes in water. The modified syringes consisted of 20 ml Sterilin syringes with the ends cut off and re-sealed with 250 µm nylon mesh, heat-welded to the cut surface thus providing a containerised system for the staining procedure (Claasen & Zasoski, 1992).

The clearing and staining procedure adopted was that of Koske & Gemma (1989). Roots were first cleared of cell cytoplasm by autoclaving in 2.5% potassium hydroxide for 15 minutes at 121 °C and 103 kPa. Pigments were then removed by soaking in a bleach solution consisting of 0.5% hydrogen peroxide and 0.175% ammonia for 1 hour. Prior to staining, roots were soaked in 1% hydrochloric acid for 1 hour. Staining of fungal structures was carried out by autoclaving the acidified roots with 0.05% Trypan Blue in acidic glycerol solution for 3 minutes at 121 °C and 103 kPa.

Clearing and staining was employed to examine roots for AM colonization. However the technique was also used in the greenhouse bioassay of burned and unburned forest soils (Chapter 4), where EM structures formed by hyphae associated with the morphotype Unknown 1 were observed in substantial portions of long roots (see section 2.3.1b). After clearing and staining, a method of assessment based on the magnified intersections method of McGonigle *et al.* (1990) was developed for quantifying this long root colonization. The method is described in Chapter 4. This method was also used for the analysis of early temporal development of EM communities after fire (Chapter 6).

### 2.4.4 Synthesis experiment involving three isolates of post-fire ascomycetes

**a) Isolation and culture of inoculants**

Isolate JM11 (*Geopyxis* sp.) – isolated from an EM root tip from a *Pinus halepensis* seedling excavated from Malesina (see Chapter 5 for locality details). After excavation on 2 May 2001, the root system was wrapped in wet kitchen towel, sealed in a plastic bag and returned to the laboratory. On 3 May 2001, ectomycorrhizal root tips were removed from the root system under a dissecting microscope. These tips were then surface sterilised in 70% ethanol for 1 minute, bleach (5% active chlorine) for 20 seconds and then washed three times in sterile water (Vralstad *et al.*, 1998). Root tips were then placed in a Petri dish on potato dextrose agar (PDA), adjusted to pH 7.4 with added chloramphenicol (made up at 0.25 g/ 50 ml ethanol and added to media at 10 ml/ litre before autoclaving) and streptomycin (made up at 1 g/ 750 ml sterile water and added to media at 100 ml/litre). Plates were observed under a dissecting microscope on a daily basis and suspected contaminant organisms removed or transferred to new dishes as appropriate. After colonies of the desired fungus had established, the petri dishes were sealed with parafilm and returned to Scotland. On 15 May 2001, part of the expanding colony was transferred onto half-strength PDA (pH 5.6).

Isolate JM13 (*Anthracobia* cf. *macrocystis*) – isolated from spores collected from a fruitbody. On 13 December 2000, fruitbodies of *Anthracobia* cf. *macrocystis* ((Cke.) Boud.)

were collected from burned forest areas near Malesina (see Chapter 5 for locality details). In the field, fruitbodies were stuck individually to the inside surface of empty sterile Petri lids with lanolin. The Petris were sealed with parafilm and returned to the laboratory. On 14 December 2000 the fruitbodies were transferred to fresh lids that were then placed over half-strength PDA (pH 7.4) with added chloramphenicol (as above) and streptomycin (as above). Ascospores were allowed to drop onto the agar surface for different periods (1-12 hours depending on the condition of the fruitbody) by transferring the lids with fruitbodies to new PDA plates. Plates were monitored for contaminant organisms as described above, and after colonies of the desired fungus had established, the petri dishes were sealed with parafilm and returned to Scotland. After three weeks parts of expanding colonies from individual Petris were transferred to a sterile vermiculite:peat mix (9:1 v/v) in separate 500ml conical flasks with half-strength PDA solution as added nutrient. Hence isolate JM13 was derived from multiple spores from a single fruitbody.

Isolate JM21 (*Byssonectria fusispora*) - isolated from spores collected from a fruitbody of *Byssonectria fusispora* (Berk.) Rogerson & Korf (syn. *Inermisia fusispora*) collected on 16 December 2000 from a burned forest site at Sofiko in the north-east Peloponnese. The method used was the same as described for isolate JM13.

**b) Molecular identification of isolate JM11**

*DNA extraction*

A square of agar measuring approximately 0.5 x 0.5 cm was cut from the expanding edge of the colony and placed in an empty Eppendorf tube and frozen in liquid nitrogen. The frozen tissue was then crushed and ground with a micropestle. The tubes were incubated at 60 °C for 45 minutes with 350 µl of 2x CTAB lysis buffer. The tubes were cooled to room temperature for 15 minutes and then 350 µl of chloroform:isoamyl alcohol (24:1) was added to each sample. Samples were vortexed briefly and centrifuged at 13 000 rpm for 10 minutes. The aqueous phase was removed from each sample and placed in a new tube. 2.5 volumes of ice-cold, pure ethanol were added and tubes were mixed by inversion. The samples were placed in a freezer at –20 °C overnight. The precipitant was pelleted by centrifugation at 13000 rpm for 30 minutes. The supernatant was discarded and the DNA pellet was washed with ice-cold 70% ethanol. The samples were centrifuged at 13000 rpm for 5 minutes. The ethanol was poured off and the pellets were left to dry for 1 hour. Each DNA pellet was then resuspended in 50 µl of TE buffer. The samples were refridgerated overnight and then heated to 60 °C for 5 minutes. Samples were then homogenised by pipetting and centrifuged at

10000 rpm to separate out any remaining debris. Two microlitres of RNAase was added to the DNA stock solution.

*PCR amplification*

The primers used were ITS1-F (Gardes & Bruns, 1993) and NL6Bmun (Egger, 1995). Used together these primers target a section of rDNA of approximately 1100 base pairs comprising the ITS1, 5.8S, ITS2 and a short section of the 28S gene. A 2 µl aliquot of stock DNA samples was included in a final reaction volume of 25 µl consisting of the following components (given as final concentrations): 1x reaction buffer (Promega); 50 µM each of dATP, dCTP, dGTP, dTTP (200µM dNTP's); 2.5mM $MgCl_2$; 0.5 µM primers; 0.025 units/µl *Taq* DNA polymerase (Promega); 2% dimethyl sulfoxide (DMSO, Anachem). The reaction mix was made up to final volume using sterile distilled water.

Each reaction was overlaid with 2 drops of mineral oil. The cycling parameters set for the DNA Thermal Cycler (Perkin Elmer) were as follows: initial denaturation at 94 °C for 2 minutes; 30 cycles of denaturation at 92 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 1 minute; final extension at 72 °C for 5 minutes; stored at 25 °C at end of reaction.

*Sequencing*

PCR products were cleaned using purifying columns (Hybaid Recovery DNA Purification Kit II) and eluted in 35 µl of elution solution. Aliquots of 5 µl of cleaned PCR product were run out against a 100 bp DNA ladder (Promega) on a 1.5% agarose gel for estimation of DNA concentration. The yield was approximately 20 ng/µl.

Both forward and reverse primers were labelled with infrared dye (IRD 700/800 MWG) and prepared for sequencing with the SequiTherm EXCEL II DNA Sequencing Kit-LC (for 25-41 cm gels). A premix of total volume 17 µl was prepared for each labelled primer/sample combination. Each of these premixes contained 7.2 µl of 3.5x SequiTherm EXCEL II Sequencing buffer, 2 µl of labelled primer (i.e, 2 pmoles), 6.8 µl DNA template (= approximately 190 fmoles) and 1 µl of SequiTherm EXCEL II DNA Polymerase.

For each primer/sample combination four 0.2 ml microcentrifuge tubes were labelled G, A, T and C and 2 µl of the appropriate SequiTherm EXCEL II LC Termination Mix added. Four microlitres of premix was added to each tube of Termination Mix and mixed thoroughly by pipetting. Each reaction was overlaid with one drop of mineral oil. The reactions were denatured at 94 °C for 5 minutes and then subjected to 30 thermocycles at the

following settings: 94 °C for 30 seconds, 50 °C for 30 seconds and 70 °C for 1 minute. A ramp of 0.4 °C/s was set between annealing and extension. Upon completion samples were stored at 4 °C.

A 6% polyacrylamide denaturing gel (41 cm x 0.2 mm depth, MWG) was prepared by mixing the following constituents: 21 g crystalline urea (42% w/v), 22 ml distilled water, 6 ml Long Ranger (50% stock solution), 6 ml TBE (10x stock solution), 333 µl APS (10% stock solution), 33 µl TEMED (added last).

Once the gel was polymerised 3 µl of Stop/Loading buffer was added to each reaction. The tubes were centrifuged briefly to separate the mineral oil from the reaction mix. The tubes were then heated at 94 °C for 5 minutes to denature the samples. Aliquots of 0.6 µl of reaction mix were loaded onto the gel. The gel was then run for 9 hours.

The labelled products were visualised using a Licor 4800 $IR^2$ automated genotyper. The sequences were checked manually. Backward sequences were reversed, complimented and aligned with forward sequences using BioEdit v5.0.9 (Hall, 1999).

*Identification*

A sequence of 829 base pairs including the ITS1, 5.8S rDNA gene, ITS2 and part of the 28S gene was obtained from isolate JM11. This was compared to sequences held in the NCBI GenBank database using the BLAST search facility. The three highest scoring significant alignments are shown in Table 2.1. All three of the closest matching accessions are in the genus *Geopyxis* and come from the same source (Vrålstad *et al.*, 1998). The source material for *Geopyxis* sp. and *G. rehmii* were air-dried herbarium specimens. The former was actually listed in the source reference as *G.* cf. *vulcanalis* (Peck) Sacc. (Vrålstad *et al.*, 1998). The *G. carbonaria* accession was derived from a culture isolated from mycorrhiza-like root tips of *Picea abies* that was shown to have an ITS sequence identical to those derived from monsporic cultures from fresh ascocarps of *Geopyxis carbonaria*. The GenBank accession sequences are shorter than that of JM11 because the latter also includes a section of the 28S gene. Those of the accessions cover the ITS1, 5.8S and ITS2 only. Within this area of the rDNA the matches are good with JM11 being separated from *Geopyxis* by a very small percentage of insertion/deletion mutations and a slightly larger, though still small, number of substitutions. However as there was no single match of 100%, isolate JM11 is considered to be a species with strong affinity to *Geopyxis*.

Table 2.1 Three highest scoring significant sequence alignments derived from a BLAST search of GenBank with an 829 base pair sequence from isolate JM11 that includes the ITS1, 5.8S rDNA and ITS2 regions.

| Score | GenBank Accession | Definition | Accession length (base pairs) | Alignment identities[a] | Alignment gaps[b] |
|---|---|---|---|---|---|
| 930 | Z96992 | *Geopyxis* sp. 5.8S rRNA gene and ITS1 and ITS2 DNA (isolate 2156.H) | 571 | 550/575 (95%) | 8/575 (1%) |
| 924 | Z96991 | *Geopyxis rehmii* 5.8S rRNA gene and ITS1 and ITS2 DNA (isolate 2216.H) | 558 | 490/498 (98%) | 1/498 (0%) |
| 806 | Z96990 | *Geopyxis carbonaria* 5.8S rRNA gene and ITS1 and ITS2 DNA (isolate 1942.M) | 552 | 472/492 (95%) | 2/492 (0%) |

[a] Number of matching bases
[b] number of alignment gaps (insertion/deletion mutations)

**c) Synthesis**

Seed of *Cistus creticus* was collected from an unburned forest area adjacent to the burned area at Malesina. Hence the host seed originates from the same provenance as two of the fungal isolates (JM11 and JM13).

The method for synthesis was modified from Mason (1980). On 5 December 2001 100 seeds were placed in Universal bottles. The bottles were half filled with 30% $H_2O_2$, sealed and placed in an agitator for 10 minutes. At the end of this period the bottles were filled to the brim with sterile distilled $H_2O$. Seed that floated to the surface was collected with a sterile loop and placed on water agar (10 g/L) in 9 cm Petri dishes. The Petris were sealed and then placed in a growth cabinet at 15 °C under continuous light (Gro-lux light tubes). The seed was checked under a dissecting microscope every other day for fungal contamination. Any contaminated seeds were removed using sterile procedures.

On 13 December 2001, the medium solution was prepared as follows. 3.9 g of Potato Dextrose Agar (PDA) was heated in 100 ml of distilled water in a water bath to dissolve the nutrient fraction. The agar was allowed to settle and the nutrient solution was then poured off. This was made up to 1 litre with distilled water and Ingestads solution, a balanced nutrient solution with NPK levels set at 30, 5, 17 ppm respectively. 180 ml of the medium solution and 300 ml of vermiculite:peat mixture (9:1 v/v) were added to 500 ml conical flasks. These were plugged with cotton wool and covered with aluminium foil and autoclaved at 121 °C and 103kPa for 30 minutes.

Sterilin tubes (30 ml) were prepared by piercing a hole in the middle of the lid using a heated needle. The holes were then plugged with sterilised cotton wool (Figure 2.1). The tubes were packed with approximately 10 ml of the sterile medium. *Geopyxis* sp. inoculum was added to the tubes as two sections of agar measuring 3 x 3 mm cut from the expanding edge of the colony. For *Anthracobia* and *Bysonnectria* approximately 5 ml of V:P culture was added to each tube. Five tubes were inoculated for each isolate. The inoculum was then covered with a layer of approximately 5 ml of sterile medium and packed with a sterilised rod. A hole was made in the centre of the packed medium and a single sterile seedling planted into each hole using a sterile wire loop.

The tubes were placed in a growth cabinet at 20 °C under continuous light supplied by a battery of Gro-lux flourescent tubes. After two weeks the light regime was switched to a 16-hour light/8-hour dark cycle. The tubes were watered once weekly with 10 ml of sterile distilled water.

On 23 May 2002, the seedlings were recovered from the tubes and washed out under running tap water. The root systems were checked under a dissecting microscope for the presence of ectomycorrhizal root tips. They were then cut into fragments of approximately 1 cm in length and cleared and stained by the method described in section 2.4.3. Cleared and stained roots were mounted in PVLG and examined under the compound microscope.

## 2.5 Results and discussion

### 2.5.1 Ectomycorrhizas

Naturally establishing *Cistus* seedlings appear to form ectomycorrhizal associations with a wide range of fungi including both Ascomycetes and Basidiomycetes. A total of 30 morphotypes was recorded (Table 2.2). Descriptions and photographs of all morphotypes are included in Appendix 2.

Figure 2.1 Growth system used for aseptic synthesis of three fungal isolates with *Cistus creticus* seedlings.

Previous emphasis has been on the association between *Cistus* and species of *Tuber*. The present results clearly indicate that *Tuber*, while important, is only one part of a community of EM fungi that associate with *Cistus* in nature. The most common morphotypes found were the 'E-strain' and an unknown fungus ('Unknown 1') that both form weak ectomycorrhizas with *Cistus creticus*.

### *a) E-strain (Appendix A2.10)*

E-strain refers to an aggregate of fungi that form morphologically similar morphotypes. These have been shown to be important symbionts in burned *Pinus halepensis* forests (Torres & Honrubia, 1997) and have also been described from a wide variety of nursery and forest conditions.

Recent culturing and molecular analyses have discriminated several fungal species within the E-strain group that have been placed in the genus *Wilcoxina* (Egger *et al.*, 1991; Egger & Fortin, 1990; Yang & Korf, 1985). However, typical E-strain morphology has also been described from morphotypes formed by *Humaria hemisphaerioides* and *Tricharina gilva*

with *Picea sitchensis* (Ingleby *et al.*, 1990), *Sphaerosporella brunnea* with *Pinus banksiana* (Danielson, 1984a) and *Balsamia alba* with *Pinus jeffreyi* (Palfner & Agerer, 1998). It is likely that the term E-strain that has been used to describe numerous fungi forming ectendomycorrhizal morphotypes and that has become almost synonymous with the genus *Wilcoxina*, encapsulates a larger group of fungi within the Pezizales.

Table 2.2 Ectomycorrhizal morphotypes associated with *Cistus creticus* seedlings

| Identity | Morphotype code | Appendix number |
|---|---|---|
| Ascomycete 1 | JMCc17 | A2.1 |
| Ascomycete 2 | JMCc55 | A2.2 |
| Bankeroid 1 | JMCc36 | A2.3 |
| Basidiomycete 1 | JMCc29 | A2.4 |
| Basidiomycete 2 | JMCc47 | A2.5 |
| Basidiomycete 3 | JMCc50 | A2.6 |
| Basidiomycete 4 | JMCc57 | A2.7 |
| Basidiomycete 5 | JMCc33 | A2.8 |
| *Cenococcum geophilum* | JMCc11 | A2.9 |
| E-strain | JMCc31 | A2.10 |
| *Genea*-like | JMCc51 | A2.11 |
| *Inocybe* 1 | JMCc46 | A2.12 |
| *Inocybe* 2 | JMCc34 | A2.13 |
| *Thelephora terrestris* | JMCc49 | A2.14 |
| Thelephoroid 1 | JMCc39 | A2.15 |
| Thelephoroid 2 | JMCc37 | A2.16 |
| *Tricholoma* 1 | JMCc48 | A2.17 |
| *Tuber* 1 | JMCc38 | A2.18 |
| *Tuber* 2 | JMCc24 | A2.19 |
| *Tuber* 3 | JMCc32 | A2.20 |
| Unknown 1 | JMCc01 | A2.21 |
| Unknown 2 | JMCc45 | A2.22 |
| Unknown 4 | JMCc35 | A2.23 |
| Unknown 5 | JMCc44 | A2.24 |
| Unknown 6 | JMCc42 | A2.25 |
| Unknown 7 | JMCc56 | A2.26 |
| Unknown 9 | JMCc41 | A2.27 |
| Unknown 11 | JMCc52 | A2.28 |
| Unknown 12 | JMCc53 | A2.29 |
| Unknown 14 | JMCc40 | A2.30 |

E-strain morphotypes are characterised by a weak mantle composed of thickened hyphae, presence of a Hartig net and varying degrees of intracellular penetration of host cortical cells by hyphae. The E-strain morphotype reported in this study did not exhibit intracellular penetration. It has long been suggested that intracellular penetration by E-strain fungi is a

host-determined response. Indeed, it appears that the formation of intracellular haustoria is largely confined to associations formed with *Pinus* and *Larix* species (Laiho, 1965; Yu *et al.*, 2001). Recent structural studies have shown that the morphology of mycorrhizas formed by the typical E-strain fungus *Wilcoxina mikolae* var. *mikolae* ranges from ectendo- to ectomycorrhizal depending on the host species (Scales & Peterson, 1991a, 1991b). In association with *Pinus banksiana* this fungus formed ectendomycorrhizas with a mantle of variable width, Hartig net and extensive intracellular colonization. With *Picea mariana* and *Betula alleghaniensis* on the other hand, the same fungal isolate formed ectomycorrhizas with a well-developed Hartig net but no intracellular penetration. The mantle was thin and discontinuous in *Picea mariana* and thick and well developed in *Betula alleghaniensis*.

***b) Unknown 1*** *(Appendix 2.21)*

Unknown 1 and E-strain were morphologically similar in that they both formed types of weak ectomycorrhizas in which the Hartig net was developed but the mantle was reduced. However, they differed sufficiently to allow separation after some practice.

With Unknown 1 there was little alteration of the underlying root – i.e., root tips were often not particularly swollen unlike the E-strain morphotype in which root shape was usually substantially altered. In quantifying levels of colonization under the dissecting microscope the two types could also be differentiated by the darker colour of the E-strain which was the result of the mantle being more developed than in Unknown 1. In Unknown 1, a mantle was often virtually absent.

Viewed with a compound microscope, emanating hyphae differed from each other morphologically. E-Strain hyphae were relatively straight-walled and distinguished by the presence of verrucose wall ornamentation. Unknown 1 hyphae were smooth-walled but much more irregular in outline and more tortuous in habit. The mantle or root surface hyphae also differed. In the E-Strain morphotype these were characteristically pinched at the septa and swollen in between. This was much less apparent in Unknown 1.

Observations of cleared and stained roots of seedlings grown in bioassays of forest soils showed that the hyphae colonizing long roots were the same as those forming the morphotype Unknown 1 on short roots (Chapter 4). Long root colonization was characterised by hyphae running along the root surface, coalescing in places to form mantle-like patches and also running through the interstitial spaces within the root cortex forming Hartig-net-like structures around some cells. On both short and long roots hyphae occurring on the root surface were 6-10 μm diameter, smooth walled, unmelanised and irregular in outline.

Cytoplasmic disjunctions occurred frequently. These were characterised by cytoplasm on one side of a septum appearing granular and with many inclusions that stained darkly in cotton blue while on the other side it was markedly less granular and without inclusions.

This pattern of colonization is unusual among ectomycorrhizal fungi. A reduced mantle is a common feature of ectomycorrhizal or ectendomycorrhizal colonization associated with E-Strain fungi as noted above, but a mantle is rarely completely absent. These fungi also tend to have a well-developed, continuous Hartig net extending throughout the cortical layers. The Unknown 1 morphotype tended to have a rather discontinous Hartig net and lacked a true mantle though hyphae did coalesce in places. Similar patterns of colonization have been reported in associations between *Pinus contorta* and some species of post-fire ascomycetes (Egger & Paden, 1986b). However, results of the synthesis experiment with three species of post-fire ascomycete (reported below) suggest that these fungi do not behave in the same way with *Cistus creticus* and Unknown 1 is not attributable to any of them.

The pattern of colonization observed in Unknown 1 also has affinities to that seen in associations between species of *Helianthemum* and desert truffles in the genera *Terfezia* and *Tirmania* where a Hartig net is formed but no mantle (Dexheimer *et al.*, 1985; Fortas & Chevalier, 1992; Morte *et al.*, 1994; Roth-Bejerano *et al.*, 1990). Only *Terfezia arenaria* and *T. fanfani* have been recorded so far from Greece, both associated with *Helianthemum guttatum* (Zervakis *et al.*, 1999). However, conditions there are widely suitable and it is likely that more species will be added to the checklist in time. Species of *Helianthemum*, including *H. guttatum* are common associates of *Pinus halepensis* forests in Greece and often co-occur with *Cistus* after fires. The potential host range of *Terfezia* species has recently been extended from *Helianthemum* by the discovery of *Terfezia pfeilii* colonizing roots of the watermelon plant *Citrellus vulgaris* (KaganZur *et al.*, 1999). As many ectomycorrhizal fungi have low specificity for host species, *Terfezia* and related fungi may well also colonize *Cistus* seedlings. There is a need to investigate root colonization in *Helianthemum* after fires to see if the same structural patterns as seen in Unknown 1 are observed and whether these are formed by species of *Terfezia*. It will also be instructive to attempt synthesis of ectomycorrhizas between *Cistus* and desert truffles.

A third possible candidate for the identity of Unknown 1 is monokaryotic strains of *Tuber* species. High levels of intraspecific variation in colonizing potential and mycorrhizal structure can occur as reported for *Laccaria bicolor* in association with *Pinus banksiana* (Kropp *et al.*, 1987; Wong *et al.*, 1990; Wong *et al.*, 1989) and *Pisolithus tinctorius* (syn. *arhizus*) in association with *Pinus pinaster*, *P. banksiana* (Lamhamedi *et al.*, 1990) and *Eucalyptus grandis* (Burgess *et al.*, 1994). In general these studies found that there was

variation within groups of both monokaryotic and dikaryotic strains tested as well as variation between monokaryons and dikaryons. Strains varied in mantle thickness, colour and texture. Some strains formed no mantle at all but did form Hartig net. Kropp *et al.*, 1987) reported that one monokaryotic strain tested "...formed no mycorrhizas, although microscopic examination of the one root with questionable morphology revealed Hartig net around several cells." Significantly, Wong *et al.* (1989) found that other strains showed a preference for colonizing long roots rather than short roots.

Fully developed ectomycorrhizas of *Tuber* species are generally considered to be formed only by dikaryotic hyphae (Giovannetti *et al.*, 1994). This view is based largely on ultra-structural studies of fully formed *Tuber melanosporum* mycorrhizas showing that hyphae forming the mantle and Hartig net were bi- or multinucleate (Fasolo-Bonfante & Brunel, 1972). However, Gardes *et al.* (1990), investigating the partnership of *Laccaria bicolor* and *Pinus banksiana*, have shown that fusion between monokaryotic and dikaryotic hyphae can take place on roots once formation of ectomycorrhizas has already been initiated. It seems at least possible that monokaryotic hyphae could colonize roots in a limited way and then fuse to form dikaryotes later. Thus the presence of dikaryotic hyphae in ectomycorrhizal root tips may not necessarily mean that these hyphae colonized the root in the first place.

The hyphae of Unknown 1 are somewhat similar in morphology to those of the *Tuber* morphotypes described below and in the relevant appendices. What we may be seeing in Unknown 1 is limited colonization by monokaryotic hyphae. Synthesis experiments between *Cistus* seedlings and monokaryotic and dikaryotic strains of *Tuber* species are needed to test this hypothesis.

### *c) **Tuber** (Appendices A2.18, A2.19 and A2.20)*

Numerous species of *Tuber* have been shown to form ectomycorrhizas with *Cistus* seedlings in pure culture synthesis (Giovannetti & Fontana, 1982) and it is not surprising to have recorded three different *Tuber* morphotypes during the course of this study. The three morphotypes were identified as *Tuber* on the basis of their distinctive pseudoparenchymal mantles with epidermoid cells. *Tuber* 1 and *Tuber* 2 were both endowed with straight, unbranched, awl-shaped cystidia but differed from each other in that *Tuber* 1 had a pronounced hyphal reticulum several layers thick overlying the mantle while *Tuber 2* had no hyphal reticulum. *Tuber* 3 was similar to *Tuber* 1 in having a hyphal reticulum but differed in lacking cystidia and in being a pale brown colour as opposed to the sandy-orange of *Tuber* 1.

Several species of *Tuber* are known to form epidermoid cells in the outer mantle. Of these only *T. borchii*, *T. magnatum*, *T. brumale* and *T. puberulum* also have straight, unramified cystidia (Zambonelli *et al.*, 1993, 1995). It was not possible to determine which of these species, if any, were responsible for *Tuber* 1 or *Tuber* 2 however by general comparison, *Tuber* 1 appeared very close to *Tuber borchii*. *Tuber* 3 resembles morphotypes formed by *T. rufum* with *Corylus avellana* in generally lacking cystidia altogether and having a hyphal net on the surface of the mantle proper (Rauscher *et al.*, 1995). *Tuber rufum* is the only *Tuber* species considered to lack cystidia (Rauscher *et al.*, 1996).

While it has been generally considered that the arrangement and organisation of the fungal structures in mycorrhizas are species specific and well conserved (Agerer, 1991) it has been demonstrated recently that there is some intraspecific variation in the morphology of *Tuber* mycorrhizas (Giomaro *et al.*, 2000). However, variation in that study was largely confined to differences in the shape of hyphal cells forming the mantle. In the present study, the *Tuber* morphotypes were differentiated on characters other than mantle morphology such as presence of cystidia and a hyphal reticulum.

Straight, awl-shaped setae with basal septa were very occasionally observed emanating from some hyphae on the lateral roots of field-excavated seedlings. These setae appeared to be the same as those typically formed on fully developed ectomycorrhizas formed by some species of *Tuber* and seen in *Tuber* 1 and *Tuber* 2.

*Tuber* is a group that is well-known and commercially important in Spain, France and Italy but is much less well-known and recorded in Greece. Indeed, the latest checklist for Greece lists only three species: *T. aestivum* Vittad., *T. genadii* Pat. and *T. melanosporum* Vittad. This is unusual as conditions typically associated with truffle production elsewhere are common in Greece. Truffles generally require shallow, stony, well-drained, calcareous soils of high pH within a Mediterranean-type climate of low to moderate seasonal rainfall that occurs during winter and spring (Giovannetti *et al.*, 1994). Such conditions are common within the Mediterranean zone of Greece along with many of the typical host plants associated with *Tuber* such as *Quercus* and *Cistus*. The apparent paucity of truffles in Greece is likely to be the result of a very small pool of active mycologists and a lack of tradition and culture associated with truffle consumption and production.

### *d) Inocybe (Appendices A2.12, A2.13)*

Two different morphotypes were identified by their distinctive hyphae that have proportionately large clamp connections (often equal in diameter to the hyphae). These

differed from each other in mantle morphology in that *Inocybe* sp. 2 lacked a gelatinous matrix placing it within *Inocybe* sub-genus *Inocybe* (Beenken *et al.*, 1996). The presence of a gelatinous matrix in *Inocybe* sp. 1 places it within *Inocybe* sub-genus *Mallocybe*.

***e) Thelephora terrestris*** *(Appendix A2.14)*

The identity of this morphotype in nature was confirmed by comparison with ectomycorrhizas formed in pot-grown *Cistus creticus* that had picked up *Thelephora terrestris* in a glasshouse in Scotland. *T. terrestris* is a common greenhouse contaminant and is generally considered to be a classic early-stage fungus. The morphology of this morphotype closely resembles that described for *Thelephora terrestris* with *Picea sitchensis* (Ingleby *et al.*, 1990).

***f) Tricholoma 1*** *(Appendix A2.17)*

This was identified on the basis of its very numerous, very compact, undifferentiated, white rhizomorphs (Agerer, 1987).

***g) Cenococcum geophilum*** *(Appendix A2.9)*

This is one of the most widely reported ectomycorrhizal symbionts and is easily identified by its black granular mantle with radially arranged clusters of cells and distinctive dark, thick-walled emanating hyphae (Ingleby *et al.*, 1990).

***h) Genea-like*** *(Appendix A2.11)*

The tip morphology, light brown, thick-walled emanating hyphae with inflated tips and the angular cells of the pseudoparenchymatous outer mantle of this morphotype are very similar to descriptions of *Genea verrucosa* Vitt. + *Quercus* sp. (Jakucs *et al.*, 1998) and *Genea hispidula* Berk. et Br. + *Fagus sylvatica* (Brand, 1991).

***i) Thelephoroid*** *(Appendices A2.15 and A2.16)*

Two morphotypes were nominated as Thelephoroid due to their similarities to morphotypes formed by species of Thelephoraceae on the basis of microscopic hyphal and mantle characteristics (Agerer & Wiess, 1989). Thelephoroid 2 closely matches the morphotype "Piceirhiza nigra" described from *Picea abies* (Berg & Gronbach, 1988) which is thought to be formed by a species of *Tomentella*.

***j) Bankeroid*** *(Appendix A2.3)*

This closely resembles morphotypes associated with species of *Phellodon, Bankera* and *Hydnellum* in the Bankeraceae in having a plectenchymatous mantle of fairly uniform hyphae without clamp connections and compact but undifferentiated rhizomorphs (Agerer & Otto, 1997).

***k) Ascomycetes and Basidiomycetes*** *(Appendices A2.1, A2.2, A2.4 – A2.8)*

Basidiomycetes were identified by the presence of clamp connections. Ascomycete 1 was identified by the presence of Woronin bodies associated with the septa.

***l) Unknown*** *(Appendices A2.22 – A2.30)*

In the absence of clamp connections or Woronin bodies, or any other identifying features morphotypes were classified as Unknown.

### 2.5.2 Arbuscular mycorrhizas

Arbuscular mycorrhizal colonisation was observed in very few of the *Cistus creticus* seedlings examined and within those was confined to one or two roots only. However, both vesicles and arbuscules were observed suggesting that *Cistus* can form a functional relationship with glomalean fungi, though this seems to occur only very rarely under the conditions of the present investigation.

AM colonization in EM species may be a function of AM inoculum potential in soils. Seedlings of *Abies* and *Tsuga* collected from among grasses and herbs that are typical AM hosts have been reported as more likely to be colonized by AM fungi (Cazares & Trappe, 1993). The *Cistus* seedlings examined in the present study were all excavated from recently burned forest stands and there is some evidence that arbuscular mycorrhizal colonization of plants may be suppressed by wildfire (Dhillion & Anderson, 1993; Dhillion *et al.*, 1988; Vilariño & Arines, 1991). This may explain the very low occurrence of AM structures recorded here. However, a cursory examination of root colonization in a species of grass (*Brachypodium* sp.) co-occurring with *Cistus* after fires showed that even where AM fungal inoculum was available, *Cistus creticus* was generally not colonized (J. Milne, unreported observations). However, AM colonization of *Cistus* was not comprehensively investigated. While it is apparently very rare among seedlings colonizing burned forest ground it may be more ecologically significant in other habitats such as phrygana.

### 2.5.3 Post-fire ascomycetes

None of the three fungi inoculated onto *Cistus* seedlings in aseptic conditions formed any structures associated with ectomycorrhizal development.

*Geopyxis* colonized only the external surface of the host roots, forming loose wefts of hyphae at irregular intervals along the root length (Figure 2.2). Both *Anthracobia* and *Byssonectria* also formed loose wefts of hyphae at the root surface but they also occasionally penetrated cortical or epidermal cells respectively to form hyphal coils (Figures 2.3 and 2.4 respectively).

These findings differ slightly to those of Egger & Paden (1986b), who reported that in association with *Pinus contorta*, *Anthracobia maurilabra* and *A. tristis* formed a loose, highly discontinuous mantle on long and short roots with localized penetration of the root epidermis. *Geopyxis carbonaria* formed a thick, discontinuous mantle with extensive inter- and intracellular colonization of the epidermal and cortical cells. In the present study with *Cistus creticus*, neither *Anthracobia* cf. *macrocystis* nor *Geopyxis* sp. formed any mantle-like development at all. *Anthracobia* cf. *macrocystis* did show occasional localized penetration of the root epidermis and formation of some hyphal coils inside cortical cells but *Geopyxis* showed no intraradical penetration at all. *Byssonectria fusispora* formed no mantle structures but again did form occasional hyphal coils inside epidermal cells. None of the interactions observed give any indication of mycorrhizal association between these three ascomycetes and *Cistus creticus* seedlings. On the other hand, colonized seedlings did not appear to be detrimentally affected by these fungi in that there were no indications of leaf chlorosis or secondary metabolites being produced in the roots. Unfortunately there were no control seedlings against which to compare the growth and vigour of the colonized seedlings but they appeared generally healthy at the end of the synthesis period.

Figure 2.2 *Cistus creticus* with *Geopyxis* sp. A) Hyphae loosely associated with root surface (scale bar = 200 µm). B) Hyphae do not appear to enter the root epidermis (scale bar = 50 µm).

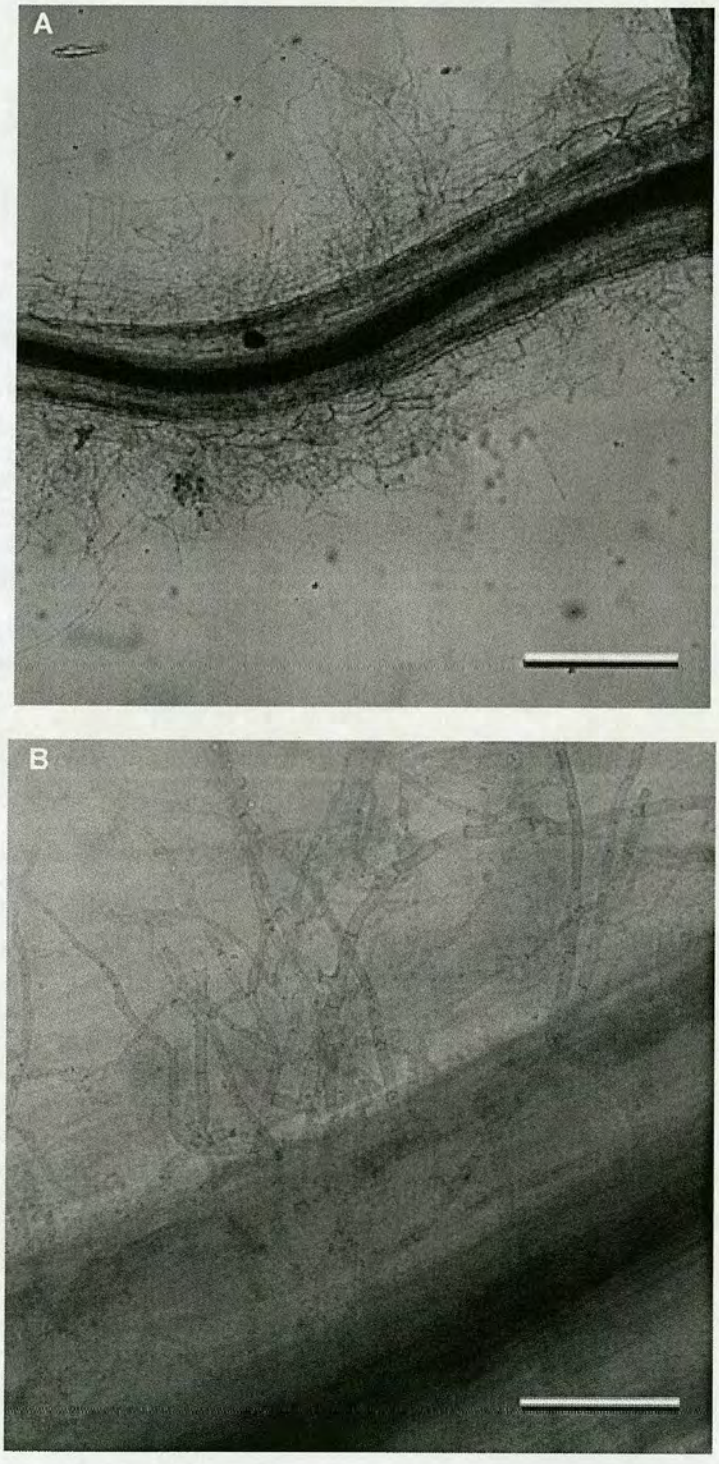


Figure 2.3 *Cistus creticus* with *Anthracobia* cf. *macrocystis*. A) Hyphae loosely associated with surface of root. Arrow indicates site of intracellular penetration (scale bar = 200 µm). B) Hyphae coiled inside root cortical cell (scale bar = 50 µm).

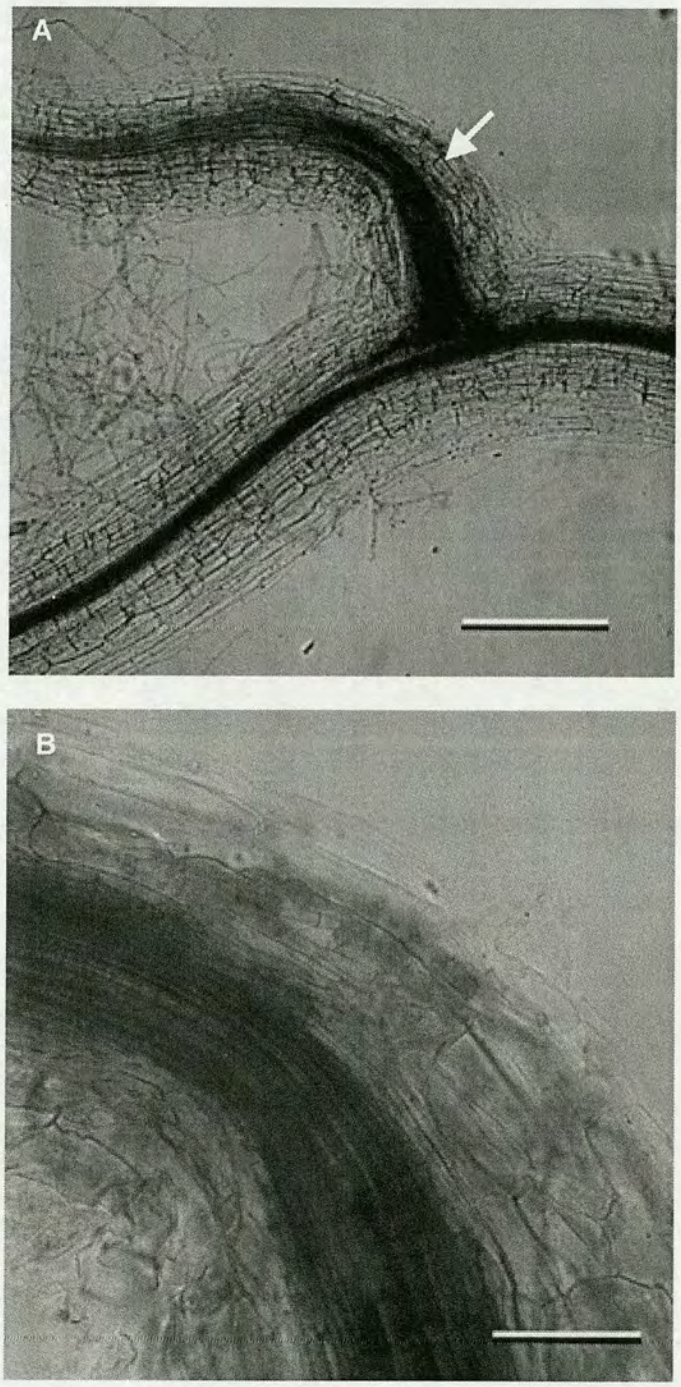
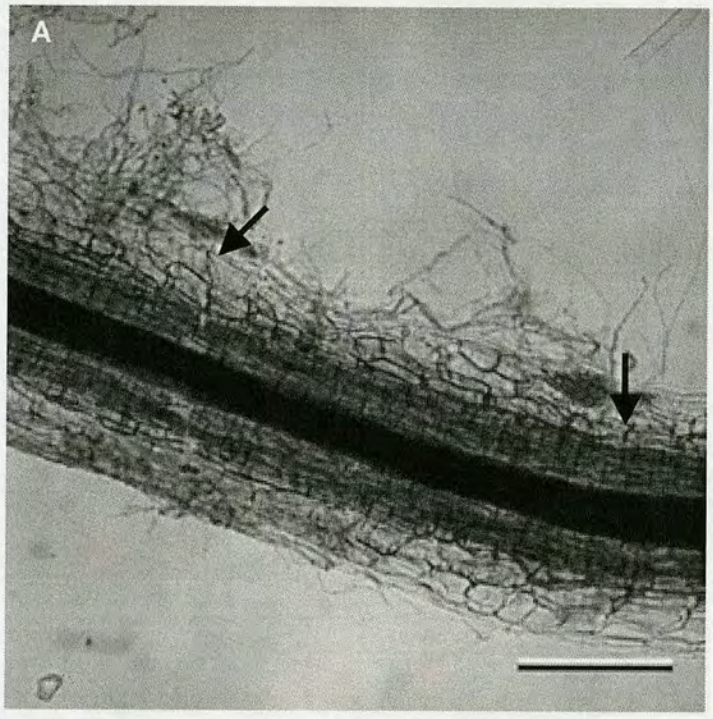
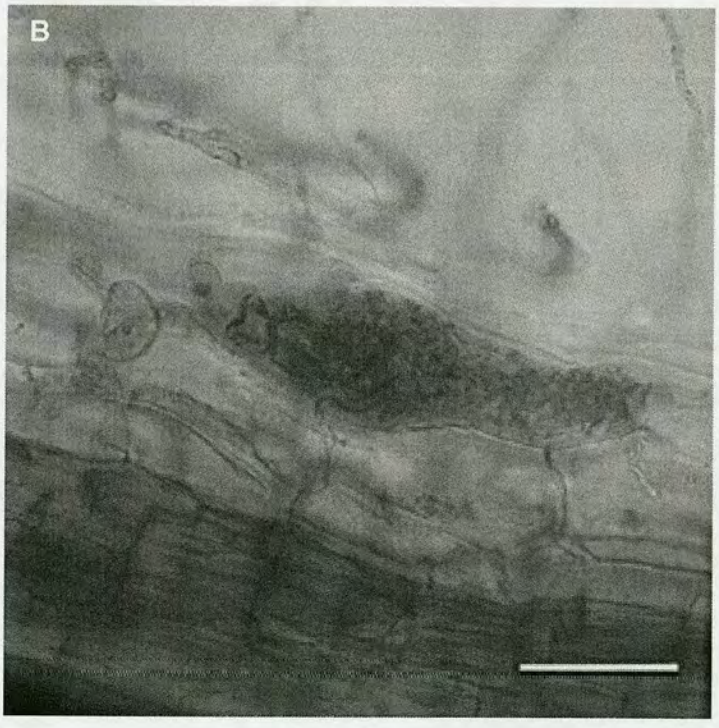


Figure 2.4 *Cistus creticus* with *Byssonectria fusispora*. A) Hyphae penetrating epidermal cells in discrete patches (arrows) (scale bar = 200 µm). B) Hyphae coiled inside root epidermal cell (scale bar = 50 µm).



## 2.6 The science of morphotyping

Traditional morphotyping of ectomycorrhizas has proved a reliable method for differentiating between different taxonomic entities and this can provide sufficient information for the quantification of ectomycorrhizal diversity in the field. However, elucidating fungal identity from morphotype descriptions remains extremely difficult in light of the relatively small number of comprehensive published descriptions. Of the estimated 5000-6000 fungal species that form ectomycorrhizas, there are currently some 140 morphotypes with systematic, comprehensive, published descriptions and photographs (Agerer, 1987-2001; Goodman *et al.*, 1996-2000; Ingleby *et al.*, 1990). In over 60 of these, the identity of the fungal symbiont remains unknown. Approximately 180 additional descriptions culled from a diverse literature are included in the DEEMY database (A DELTA-based system for characterization and DEtermination of EctoMYcorrhizae, by R. Agerer and G. Rambold, Institute for Systematic Botany, Section Mycology, University of Munchen). Again the identity of many of these fungi is unknown and many of the descriptions are incomplete.

It is within this arena of taxonomic uncertainty that molecular methods for identifying fungi directly from colonized roots have had the most immediate impact on ectomycorrhizal community studies. However it is important that traditional morphotyping techniques are not abandoned in the pursuit of a name for the fungal symbiont. Apart from being essential for sorting morphotypes prior to molecular characterisation and for quantifying relative proportions of types, observation and description of morphotype features can provide valuable clues about the ecology of the different fungi involved. An example of this can be seen in the recent attempt to interpret ecologically the nature and abundance of emanating hyphae and rhizomorphs (Agerer, 2001). Furthermore, molecular techniques require access to dedicated laboratories filled with expensive equipment. There remains a need to produce accessible morphotype descriptions for the field practitioner and for those that do not have the financial or technical resources for molecular analyses. In addition to the need for more morphotype descriptions there is a requirement for a classificatory analysis of the available descriptions to identify broad taxonomic patterns that will be useful in further developing keys to identification.

To maximise the clarity and therefore usefulness of such keys, it will be necessary to fully standardize the process of description. Since the first ectomycorrhizas were described in the late 19$^{th}$ century by Gibelli and Frank several attempts have been made to develop a comprehensive system of morphotype differentiation (Agerer, 1996). Milestones in this history include the first differentiation of rhizomorphs by Melin in 1923, Dominik's system

of characterisation based on mantle structure in cross-section published in 1956 and Chilvers and Pryor's return to the methods of Gibelli and Frank involving descriptions based on mantle structure in plan view (Chilvers & Pryor, 1965). Ingleby *et al.*, (1990) built on the work of Chilvers by incorporating his terminology for characterising mantle structure with an overall assessment of all macro- and microscopic structures to provide some of the first truly comprehensive descriptions of ectomycorrhizal morphotypes. Under the leadership of Reinhard Agerer the science of morphotype characterisation has subsequently reached a benchmark standard of minutely detailed descriptions of all aspects of ectomycorrhiza morphology accompanied by high quality photographs (Agerer, 1987-2001). However, a parallel development in morphotype description has emanated from Canada since the mid-1990's. The group at the British Columbia Ectomycorrhiza Research Network (BCERN) have published a number of excellent descriptions accompanied by photographs (Goodman *et al.*, 1996-2000). Unfortunately they reverted to the descriptive terminology for mantle structure used by Chilvers & Pryor (1965) and Ingleby *et al.* (1990). Evidently there is a need for standardization of terminology and I suggest that that of Agerer (1987-2001) be adopted as the standard as this is the most technically precise.

Considerable problems remain in the characterisation of EM communities. These reside largely in the quantification of relative abundance of individual morphotypes among samples. Where differences between morphotypes are obvious at the macroscopic level, it is relatively straight-forward to count the number of root tips associated with different fungi. Where morphotypes differ mainly in some microscopic features it becomes very difficult. It is simply not feasible to prepare and check every single root tip at high magnification to look for these cryptic characters. This problem is somewhat ameliorated by the fact that even closely similar morphotypes do take on a certain individuality after a great deal of time has been spent observing and checking them. Nonetheless, using morphotyping techniques alone, it is inevitable that some misrecording takes place. Most studies of EM communities that have used molecular tools to identify the fungi involved, rely on morphotyping to quantify and separate different morphotypes in the first place. Typically, a small sub-set of root tips of each morphotype is then selected for molecular analysis. Evidently the possibility of misrecording at the morphotyping stage still exists although there is perhaps a greater chance of detecting this through amplification of different molecular signals within the morphotype sub-samples. The smaller the sub-samples however, the smaller the chance of picking up these mistakes. Undoubtedly, the future of research into mycorrhizal ecology lies in a marriage of traditional and novel techniques.

# Chapter 3 – Spatial variation in colonisation of *Cistus* seedlings by ectomycorrhizal fungi in Aleppo pine (*Pinus halepensis* Mill.) forests after fires.

## 3.1 Introduction

In studies conducted to date, the most prominent effect of forest fires on the mycorrhizal fungal community appears to be the fragmentation of a more or less continuous mycelial network consisting of a few dominant and many rare species, mostly within the Basidiomycetes (Gardes & Bruns, 1996; Horton *et al.*, 1998; Stendell *et al.*, 1999). At least in one case this is reported to result in a random distribution of point source inocula in the months immediately following the fire (Grogan *et al.*, 2000a). In other cases however, at least some species of fungi are reported to be uniformly distributed across burned sites (Horton *et al.*, 1998; Torres & Honrubia, 1997). This chapter reports the results of field observations that were conducted to assess the spatial distribution of ectomycorrhizal fungal inoculum in recently burned forest soils.

Post-fire distribution of ectomycorrhizal fungal inoculum is likely to be influenced by a combination of environmental factors that help to protect fungi from the harmful effects of fire or promote their recovery after fires and the species attributes of individual fungi and their hosts. It has been suggested that after disturbances, plant species that recover quickly by resprouting may act as refugia for the mycorrhizal fungi required by species that germinate from seed (Perry *et al.*, 1989). It has been suggested that this influence may be exerted through alteration of soil biological activity in terms of nutrient status (Borchers & Perry, 1990) or through the maintenance of fungal populations that can only colonize new seedlings from ectomycorrhizas already established on living roots (Kranabetter *et al.*, 1999).

In Mediterranean pine forests, many species of understorey shrubs quickly resprout from underground storage organs after fires. Among the most abundant of these are *Quercus coccifera* and *Pistacia lentiscus* which are ectomycorrhizal and arbuscular mycorrhizal respectively (Puppi & Tartaglini, 1991). New roots produced from the ligno-tuber of *Quercus coccifera* may provide early sources of carbohydrates required for the regeneration of ectomycorrhizal fungi and thus seedlings establishing close to resprouting stems may harbour a greater diversity and abundance of ectomycorrhizas than seedlings establishing close to arbuscular mycorrhizal shrubs or in the gaps between shrubs. On the other hand, the soil around resprouting shrubs, regardless of their mycorrhizal status may be beneficial to

ectomycorrhizal fungi. In this case it is expected that seedlings establishing close to either *Quercus coccifera* or *Pistacia lentiscus* would have a greater diversity and abundance of ectomycorrhizas compared to seedlings establishing in gaps.

Another important factor in determining post-fire patterns of ectomycorrhizal colonization may be the distribution of ectomycorrhizal fungi in the soil profile. Even in intense fires, heating of soil is confined largely to the upper few centimetres of the profile (Debano *et al.*, 1998; Rundel, 1983). Thus, while surface accumulations of litter and associated micro-organisms, including mycorrhiza-forming fungi, are consumed by fire, sub-surface soil layers may be less affected. Some fungi may colonize seedlings once the root system has penetrated the lethally affected upper soil layers. In such cases we might expect to observe some vertical stratification in root colonization by different species of fungi. No such vertical structure in the post-fire ectomycorrhizal fungal community was found in Californian *Pinus muricata* forest (Grogan *et al.*, 2000a) where most of the fungi successfully colonized *Pinus* seedlings throughout the upper 20 cm of soil. However, the abundance of colonization at different depths was not quantified and also the study was carried out 1.5 years after the fire. By this time the distribution of fungi within individual root systems may have been determined more by secondary infections from established mycorrhizas than by initial inoculum sources.

The aims of this study were: 1) to assess the effect of proximity to resprouting shrubs on colonization of naturally establishing *Cistus* seedlings by ectomycorrhizal fungi; 2) to assess the vertical spatial distribution of mycorrhizal morphotypes within the root systems of seedlings.

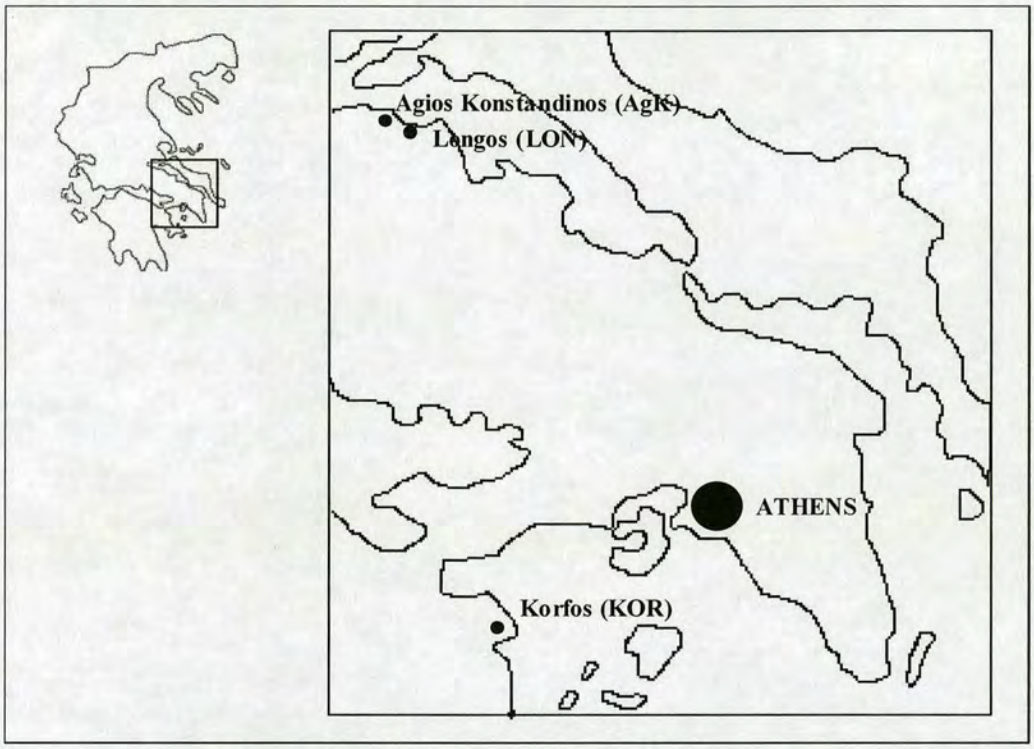
## 3.2 Methods

### 3.2.1 Study sites

Following wildfires in August 1999, three geographically separated burned forest sites were selected. The sites were at Longos (LON) ($38^0$ 44' N, $22^0$ 56' E) Agios Konstandinos (AgK) ($38^0$ 45' N, 220 55' E) and Korfos (KOR) ($37^0$ 45' N, $23^0$ 10' E) (Figure 3.1). Before the fires, each site comprised mature stands of *Pinus halepensis* with a mixed understorey of woody shrubs (see below). At all three sites the fires were intense, resulting in complete scorching of tree crowns and tree death, complete consumption of understorey shrub leaf biomass and complete consumption of litter. Resprouting of understorey shrubs began soon after the fires. After the first autumn rains in October, germination of seeds resulted in the

development of a low-density ground layer dominated by *Cistus* seedlings and including *Fumana thymifolia*, another species in the Cistaceae. Both *Cistus creticus* and *Cistus salvifolius* L. were present with *Cistus creticus* being the more abundant of the two. *Pinus halepensis* seedlings were also present at very low densities.

Figure 3.1 Map showing location of sampling sites in central Greece




In the November following the fires, the sites were characterised by recording slope, aspect, percentage cover of rocks on the soil surface, stand density and tree diameter at breast height (DBH) (Table 3.1). The latter three parameters were measured at a number of randomly selected locations along established baselines (see below) at each site (Table 3.1). Percentage cover of rocks was estimated as the mean proportion of a 5 m length of tape measure intercepted by rocks and stones greater than 1 cm in diameter. The tape measure was laid parallel to the contour of the slope at each shrub location. Stand density was recorded as the mean number of mature trees (> 9.5 cm DBH) occurring within a 10 m radius of selected shrub locations. Diameter at breast height was recorded as the mean DBH of the mature trees occurring within a 10 m radius of the shrub locations. The occurrence of regenerating

understorey plants was also recorded. The abundance of each species was recorded on the DAFOR scale according to the criteria laid out in Table 3.2 and is presented in Table 3.3.

### 3.2.2 Sampling

Seedlings were collected in February, 2000. One baseline, 150-m long, was established perpendicular to the slope at each site. At randomly selected locations along the baseline, five resprouting individual ectomycorrhizal shrubs ["EM-shrub"] (*Quercus coccifera*) and five non-ectomycorrhizal shrubs ["Non-EM-shrub"] (either *Pistacia lentiscus* or *Phyllirea latifolia*) were selected. In addition, five open areas ["Open"], defined as being greater than one metre from any resprouting shrub, were selected at random locations along the baselines. EM-shrub, Non-EM-shrub and Open are henceforth referred to as 'microsites'.

Table 3.1 Site characteristics at Longos (LON), Agios Konstandinos (AgK) and Korfos (KOR). Figures indicate mean per site (± se) based on *n* random locations along the 150-m baselines.

| | LON | AgK | KOR |
|---|---|---|---|
| n | 9 | 8 | 6 |
| Slope | Steep (>30$^0$) | Steep (>30$^0$) | Moderate (~20$^0$) |
| Aspect | SE | N | S |
| % cover of rocks on surface | 5 (±1) | 24 (±3) | 25 (±1) |
| Stand density (No. trees ha$^{-1}$) | 386 (±55) | 147 (±18) | 276 (±28) |
| DBH (cm) | 22 (±1) | 23 (±1) | 21 (±1) |
| Resprouting shrubs present | *Quercus coccifera*<br>*Pistacia lentiscus*<br>*Arbutus unedo*<br>*Clematis vitalba* | *Quercus coccifera*<br>*Pistacia lentiscus*<br>*Phyllirea latifolia*<br>*Arbutus unedo*<br>*Olea europaea* | *Quercus coccifera*<br>*Pistacia lentiscus*<br>*Phyllirea latifolia* |

One to three *Cistus* seedlings were excavated at each sampling point. It was believed at the time that all seedlings excavated were *Cistus creticus*. However, at this early stage of

development, *C. creticus* and *C. salvifolius* seedlings are very similar to each other. I cannot therefore rule out the possibility that both species were sampled.

At shrub microsites seedlings were taken from within 30 cm of resprouting stems. A total of 90 seedlings were excavated from 45 locations (5 replicate locations for each of the three 'treatments' at three sites). The seedlings excavated ranged in developmental stage from 2 to 12 true leaves. Seedlings were wrapped in absorbent paper that was then soaked in water and sealed in plastic bags in an insulated cool-box for transport back to the laboratory where they were stored at 4 $^{0}$C until further processed.

Table 3.2 Definition of DAFOR abundance terms (modified from Jerram & Drewitt (1998)).

| | |
|---|---|
| Dominant | A single species which prevails over other species in terms of the ground cover of a stand of a particular habitat. |
| Abundant | Found regularly throughout a stand of a particular habitat and contributing significantly to the estimated ground cover of that stand (>5% cover). |
| Frequent | Scattered plants or small clumps of plants found regularly throughout a stand and making a modest contribution to the estimated ground cover of that stand (<5% cover). |
| Occasional | Scattered plants generally not making a contribution to the ground cover of that stand. |
| Rare | No more than a few individual plants or clumps of a species recorded in a stand |

### 3.2.3 Assessment

In the laboratory all seedlings were carefully washed free of soil under running tap water and placed in water in Petri dishes. Seedlings were then further cleaned manually with the aid of a dissecting microscope and stored in 2% glutaraldehyde until assessment which occurred within one month of excavation.

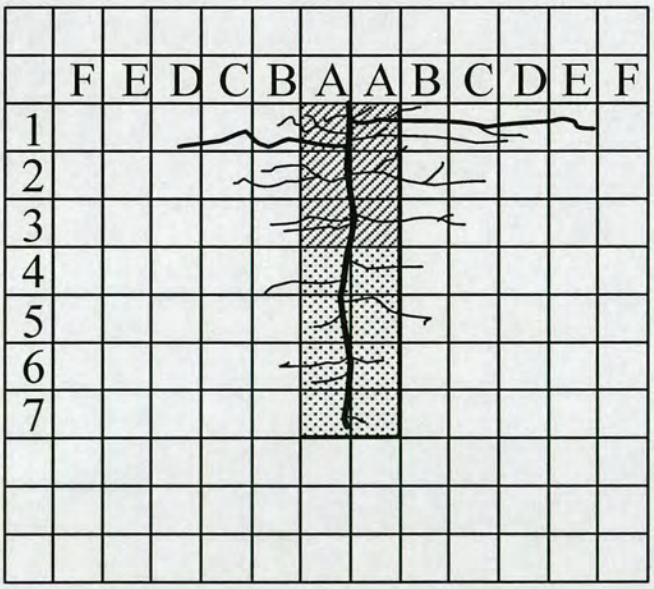
At assessment, the 75 seedlings collected from AgK and KOR were placed in a large Petri dish overlying graph paper of 1 cm grid size. Seedlings were arranged such that the seminal root was aligned with a vertical grid line and lateral roots were drawn out to lie perpendicular to the seminal root. Root tips were counted within each cell of the underlying

Table 3.3 Occurrence of understorey species at Longos (LON), Agios Konstandinos (AgK) and Korfos (KOR). (D=Dominant; A=Abundant; F=Frequent; O=Occasional; R=Rare; - = Absent. See Table 3.2 for definitions).

| | AgK1 | KOR1 | LON1 |
|---|---|---|---|
| *Arbutus andrachne* | - | - | R |
| *Arbutus unedo* | A | - | A |
| *Clematis vitalba* | - | - | F |
| *Cyclamen sp.* | - | R | A |
| *Olea europaea* | F | - | - |
| *Phyllirea latifolia* | F | D | - |
| *Pistacia lentiscus* | F | A | A |
| *Quercus coccifera* | A | A | F |
| *Smilax aspera* | A | - | - |

grid of 1 x 1 cm squares extending 7 cm down from the root collar and 6 cm away from the primary root (Figure 3.2). These distances were chosen arbitrarily to encompass the majority of sample root systems. Root tips were recorded as non-mycorrhizal or according to ectomycorrhizal morphotype using morphotyping techniques described in Chapter 2. The 15 seedlings from LON were assessed without recording vertical distribution of morphotypes on the grid.

Figure 3.2 Representation of the grid used for recording the spatial distribution of ectomycorrhizal morphotypes within root systems. Shaded area represents spatial section A used for separation of morphotypes into spatial groups. Dark shading = root depth 1-3 cm; light shading = root depth 4-7 cm; dark + light shading = root depth 1-7 cm.



For descriptive purposes, after assessment, morphotypes recovered from sites AgK and KOR were placed in spatial groups according to their horizontal and vertical distribution. Morphotypes were first separated according to the microsites in which they were present (shrub and open, shrub only, open only). Morphotypes were then further separated according to their depth distribution by placing them in one of three categories (predominantly in the top 3 cm; well-represented throughout root depth recorded; predominantly below 3 cm) (see Table 3.4 for summary of spatial groups). Only root tips occurring in spatial section A, i.e, within 1 cm horizontally either side of the primary root (see Figure 3.2), were considered in the grouping. This was because of the uncertainty of the angle of attack of lateral roots in the soil such that root tips recorded at position 1F for instance, could have been lying at position 4A in the soil (Figure 3.2). The number of root tips in each spatial grid cell colonised by any of the morphotypes within each spatial group were added together to give a composite group value for each spatial grid cell in each seedling. Composite values were then averaged across all seedlings in which any of those morphotypes occurred.

Two morphotypes (*Tuber* 3, Basidiomycete 5) found only at site LON were not placed in spatial groups as depth distribution was erroneously not recorded for seedlings collected from LON.

Table 3.4 Summary of spatial groups within which morphotypes were classified for descriptive purposes after assessment.

| | Shrub + Open | Shrub only | Open only |
|---|---|---|---|
| Root depth = 1-3 cm | Group 1 | Group 4 | Group 7 |
| Root depth = 1-7 cm | Group 2 | Group 5 | Group 8 |
| Root depth = 4-7 cm | Group 3 | Group 6 | Group 9 |

### 3.2.4 Statistical analysis

Where more than one seedling were collected from a single sampling location at AgK and KOR the root-tip counts were averaged to give a single value for each sampling location replicate. Level of colonization was expressed as 'percentage colonization' which is the proportion of total root tips colonized per seedling. In addition to percentage colonization, frequency of occurrence of individual morphotypes was expressed as the proportion of seedlings colonized. Percentage colonization, number of morphotypes per seedling and Shannon-Wiener diversity data were arcsine, square root and log transformed respectively.

Data were checked for normality using the Kolmogorov-Smirnov Test. Homogeneity of sample variance was checked using Bartlett's Test. Transformed data were subjected to two-way analyses of variance with microsite and site as fixed and random factors respectively.

## 3.3 Results

### 3.3.1 Ectomycorrhizal colonization

The general pattern of colonization observed was one of dominance at all locations by a few ascomycetes ('E-strain', *Tuber* spp., Ascomycete 1) and rare occurrence of several basidiomycetes (*Tricholoma* 1, *Inocybe* spp., Thelephoroid spp., Bankeroid 1, Basidiomycetes 1, 2 and 5) (Figure 3.3).

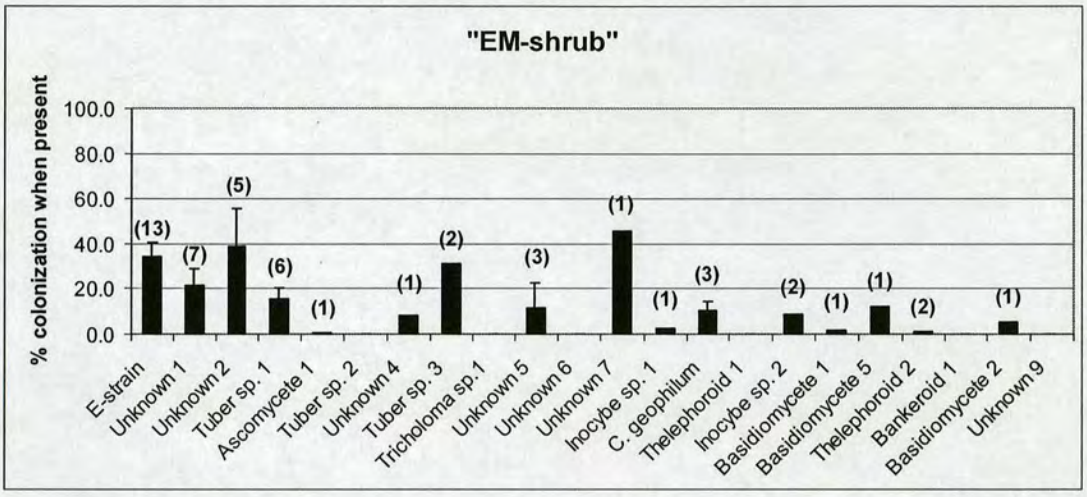
On the basis of combined overall frequency and percentage colonization, by far the most common morphotypes at all sampling locations were 'E-strain' and 'Unknown 1'(Figure 3.3). 'E-Strain' was recorded from 34 of the 45 sampling points with an average percentage colonisation where present of 35.6 % (± 4.8 s.e.). 'Unknown 1' was recorded from 18 sampling points with an average percentage colonization where present of 30.2 % (± 3.6 S.E.). *Tuber* 1 occurred at 12 of the sampling points but with a lower percentage colonization (13.4 % ± 3.3 s.e.). A few morphotypes occurred at low frequency but high percentage colonization (e.g., *Tricholoma* 1, Unknown 7). Most of the rest occurred at low frequency and low percentage colonization.

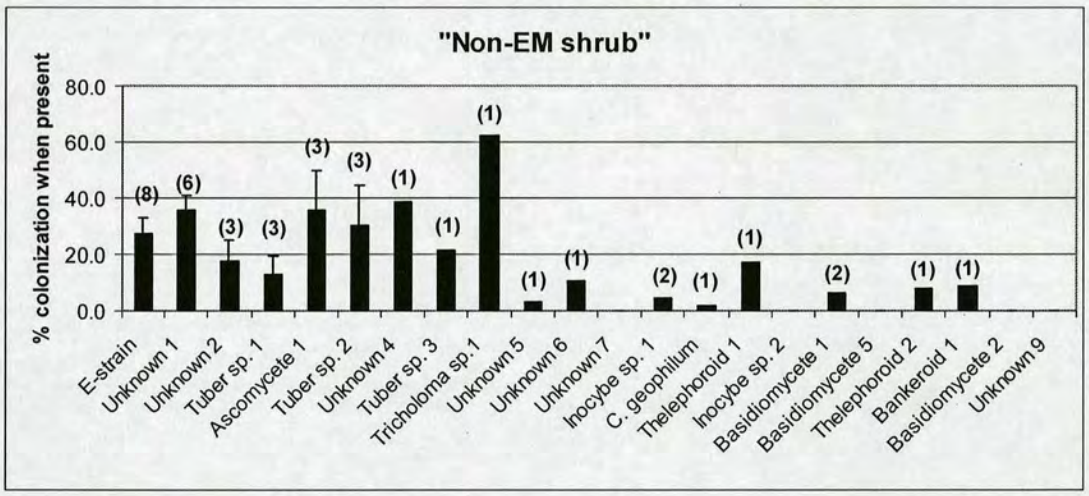
There were no significant effects of site or microsite on percentage colonization, number of morphotypes per seedling or Shannon-Wiener diversity. Table 3.5 shows the average values of these parameters at the three microsites for comparative purposes (data pooled across sites). These data indicate a general trend of lower morphotype diversity in open microsites.

Considering individual seedlings, there was no significant correlation between number of morphotypes and percentage of root tips colonized (Figure 3.4). The percentage colonization of seedlings was extremely variable. For example, percentage colonization of seedlings with a single morphotype ranged from 6.7 % to 100 % (Figure 3.4).

There were no significant relationships between seedling height or number of primary leaves and number of morphotypes per seedling or percentage of root tips colonized (Figure 3.5).

Figure 3.3 Abundance and frequency of ectomycorrhizal morphotypes associated with *Cistus* seedlings establishing in three different locations in burned *Pinus halepensis* forests. Blocks = mean % colonization when present. Figures in parentheses = frequency of occurrence out of n = 15 sampling points. Morphotypes are ranked left to right according to their overall combined frequency and abundance values.



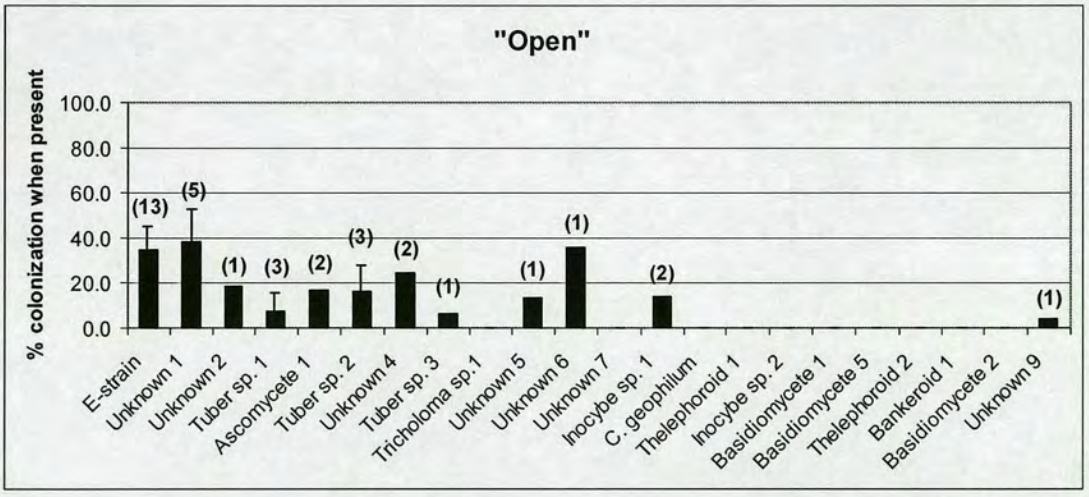



Table 3.5 Effect of microsite on total number of morphotypes and mean (± s.e.) percentage colonization, number of morphotypes per seedling and Shannon-Wiener diversity at 'EM shrub', 'Non-EM shrub' and 'Open' locations for *Cistus* seedlings excavated from recently burned forest sites.

| | EM shrub | Non-EM shrub | Open | All |
|---|---|---|---|---|
| Sample size | 15 | 15 | 15 | 45 |
| % colonization | 70.4 (± 4.9) | 61.5 (± 4.6) | 65.7 (± 6.8) | 65.9 (± 3.2) |
| Total No. morphotypes | 16 | 19 | 12 | 22 |
| No. morphotypes per seedling | 2.3 (± 0.2) | 1.9 (± 0.2) | 1.6 (± 0.1) | 1.9 (± 0.1) |
| Shannon-Wiener diversity (H') | 0.569 (± 0.09) | 0.457 (± 0.10) | 0.317 (± 0.09) | 0.448 (±0.06) |

Figure 3.4 Relationship between number of ectomycorrhizal morphotypes per seedling and percentage of root tips colonized in *Cistus* seedlings establishing in burned forest soils.

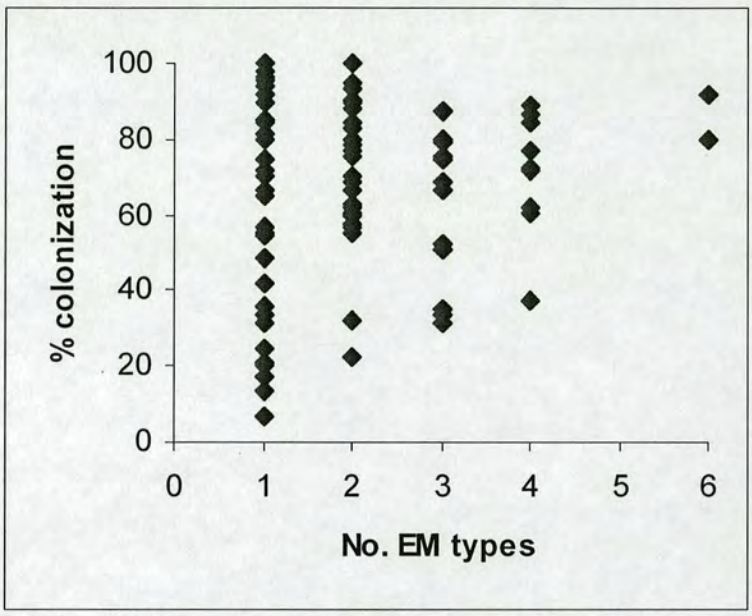
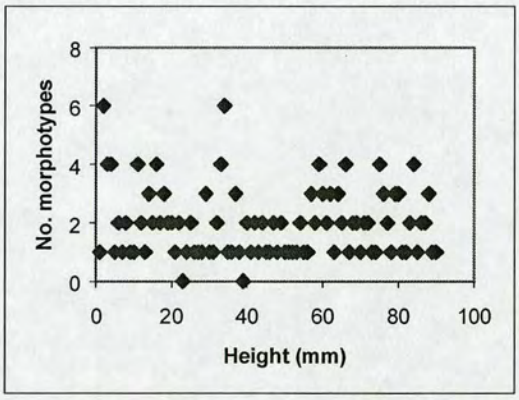
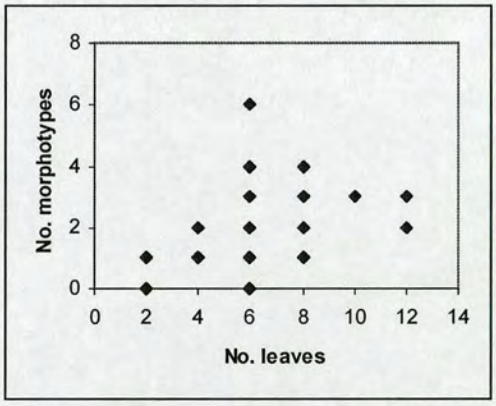
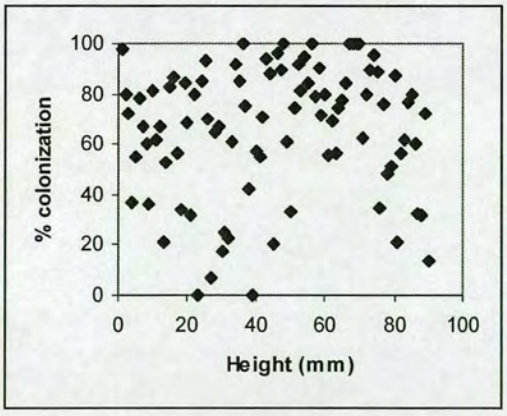
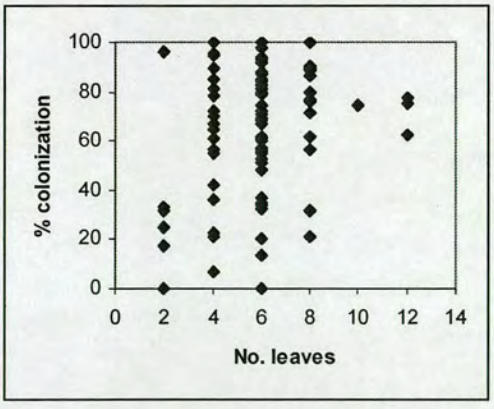



Figure 3.5 Relationship between seedling height and number of leaves and number of ectomycorrhizal morphotypes per seedling and percentage of root tips colonized in *Cistus* seedlings establishing in burned forest soils.



### 3.3.3 Spatial distribution of morphotypes

The placement of morphotypes into seven spatial groups is shown in Table 3.6. Of the total 20 morphotypes considered, 10 were found at both shrub and open locations, 9 were found only at shrub locations and one was found only at open locations though this was only on a single seedling (Table 3.6). The vertical distribution of morphotypes in Groups 1-6 is displayed in Figure 3.6. Group 7 was not included in Figure 3.6 as it contained only a single seedling.

The very common 'E-strain' and 'Unknown 1' fungi of Group 1 were found at all microsites, predominantly in the top 3 cm of root systems (Table 3.6, Figure 3.6).

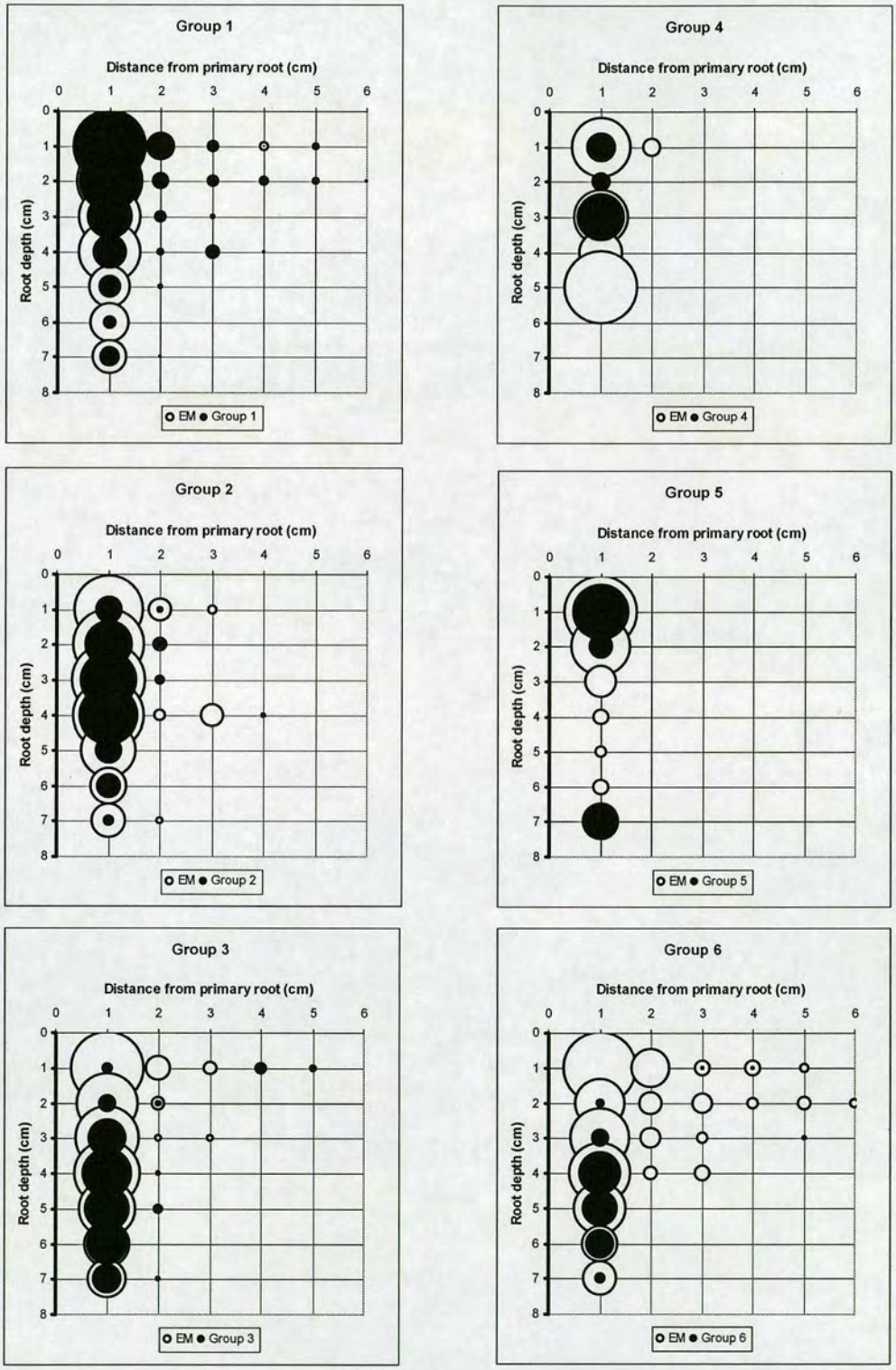
Morphotypes formed by species of *Tuber*, particularly *Tuber* 1, were an important component of the post-fire ectomycorrhizal community and were found at both "shrub" and "open" locations. Placed in Group 3, *Tuber* morphotypes were found predominantly below 3 cm (Table 3.6, Figure 3.6).

All confirmed basidiomycetes, with the sole exception of *Inocybe* 1 were confined to the shrub microsites (Table 3.6). Of the seven fungi putatively identified as basidiomycetes found only at shrub locations, two were found only at "EM-shrub", three only at "Non-EM shrub" and two were found at both (Table 3.6). However the low frequency of encounter associated with these fungi (often single seedlings) precludes further comment. Only one of the basidiomycete morphotypes (Bankeroid 1) was found predominantly in the upper 3 cm of the soil (Group 4, Table 3.5, Figure 3.6). All other confirmed basidiomycete morphotypes were found predominantly below 3 cm (Group 6, Table 3.6, Figure 3.6). The unknown morphotypes placed in Groups 2 and 5 occurred throughout the upper 7 cm of the soil profile while the only morphotype found only at open locations occurred in the upper 3 cm though this was only recorded from a single seedling (Table 3.6).

Table 3.6 Placement of morphotypes within spatial groups according to their occurrence at shrub and/or open microsites and their vertical distribution on root systems. Numbers in parentheses are the number of seedlings on which a morphotype occurred (n = 75). Within the shrub only grouping, the identity of the shrub is given (EM = ectomycorrhizal shrub (*Quercus coccifera*); Non-EM = non-ectomycorrhizal shrub (*Pistacia lentiscus* or *Phyllirea latifolia*); Both = EM and Non-EM). Basidiomycete 5 and *Tuber* 3 were not included in the spatial grouping as these two morphotypes occurred only at LON and their depth distribution was not recorded.

| | **Shrub + Open** | **Shrub only** | | **Open only** |
|---|---|---|---|---|
| **Root depth = predominantly 1-3 cm** | **Group 1**<br>E-Strain (42)<br>Unknown 1 (30) | **Group 4**<br>*C. geophilum (2)*<br>Bankeroid 1 (2) | <br>Both<br>Non-EM | **Group 7**<br>Unknown 9 (1) |
| **Root depth = 1-7 cm** | **Group 2**<br>Unknown 2 (10)<br>Unknown 4 (3)<br>Unknown 5 (7) | **Group 5**<br>Unknown 7 (2) | <br>EM | **Group 8** |
| **Root depth = predominantly 4-7 cm** | **Group 3**<br>*Inocybe* 1 (6)<br>*Tuber* 1 (19)<br>*Tuber* 2 (2)<br>Ascomycete (4)<br>Unknown 6 (3) | **Group 6**<br>*Inocybe* 2 (1)<br>*Tricholoma* 1 (1)<br>Thelephoroid 1 (1)<br>Thelephoroid 2 (3)<br>Basidiomycete 1 (4)<br>Basidiomycete 2 (1) | <br>EM<br>Non-EM<br>Non-EM<br>Both<br>Both<br>EM | **Group 9** |

Figure 3.6 Vertical distribution of root tips colonised by fungi in spatial groups 1-6 (see text and Table 3.6 for definitions of spatial groups). White circles are total number of colonized root tips (EM) and black circles are number of root tips colonized by fungi in each spatial group. The size of the circles is proportional to the arithmetic mean. The actual values and standard errors are given in Appendix 3.



## 3.4 Discussion

### 3.4.1 Morphotypes

Twenty-two morphotypes were differentiated from a total of 90 seedlings examined six months after a wildfire and four months after host seed germination. Compared to other studies this appears to be a relatively high number. Studies of naturally establishing *Pinus muricata* seedlings in burned pine forests in North America recorded 13 morphotypes from 50 seedlings during the first five months after the fire (Horton *et al.*, 1998), only seven morphotypes from 29 seedlings after one year (Baar *et al.*, 1999) and 30 morphotypes from 42 seedlings after 18 months (Grogan *et al.*, 2000a). Elsewhere, in Indonesia, 24 morphotypes were recorded from 40 *Shorea parvifolia* seedlings 9 months after logging (Ingleby *et al.*, 1998). The present study demonstrates, for the first time, that naturally establishing *Cistus* seedlings have a relatively broad receptivity towards fungal symbionts (sensu Molina *et al.* (1992)). This may be an adaptive trait that has evolved in response to the patchy distribution of many mycorrhizal fungi after fires. In the post-fire environment it may be advantageous for obligately seeding plant species to exhibit low specificity for fungal associates to ensure that mycorrhizas can readily be formed with whatever fungal inoculum is available at the establishment microsite. This may be particularly relevant in the case of small-seeded species that lack sufficient nutrient reserves to remain independent of soil minerals for very long after germination (Allsopp & Stock, 1992, 1995). For these species, early access to the enhanced nutrient acquisition offered by mycorrhizal associations may be important in determining the outcome of early seedling establishment and growth.

The early mycorrhizal fungal community in recently burned forest sites in Central Greece appears to be dominated by members of the Ascomycotina. This is consistent with the results of other studies conducted in burned forests around the world where ascomycetes were highly represented in the early post-fire stages (Baar *et al.*, 1999; Horton *et al.*, 1998; Mah *et al.*, 2001; Torres & Honrubia, 1997; Warcup, 1990). The abundance of ascomycetes may be a result of a reduction in competition from the dominant mature forest fungi due to the effects of fire. Mature undisturbed pine forests tend to be dominated by basidiomycete fungi, often members of the Russulaceae and Thelephoraceae (Stendell *et al.*, 1999). As mature forest fungi are thought to proliferate in the litter layer (Deacon *et al.*, 1983; Last *et al.*, 1983; Mason *et al.*, 1983), when this is removed by wildfire, the inoculum potential of these fungi is likely to be much reduced thus allowing other fungi to colonize.

The level of colonization among individual seedlings was highly variable with seedlings with a single morphotype ranging from less than 10% to 100% colonized. The lack of correlation between level of colonization and number of leaves or seedling height suggests that this variation is not determined by the developmental state of the seedling. It is more likely to be related to spatial variation of soil inoculum potential.

### 3.4.2 Spatial distribution

The present results suggest that fungal inoculum in the post-fire forest soil is stratified both vertically and horizontally. The commonly found E-strain morphotype and the other weak ectomycorrhizal morphotype (Unknown 1) dominated in the top 3 cm of root systems across the whole burned forest floor. Basidiomycete morphotypes were rare and largely confined to lower soil layers beneath shrubs.

The upper portion of the root systems is the oldest part and fungi colonizing these roots are likely to have been the first colonizers of these young seedlings. E-Strain and Unknown 1 were very prevalent in this zone indicating a high degree of receptivity in *Cistus* to these fungi in the early stages after germination. Very young *Cistus* seedlings have an immediate requirement for external sources of nutrients because their seeds are small and yet may not be able to support fungi with a high carbon demand at this early stage in their development. Therefore it may be an advantage to the seedlings to be able to form associations with fungi that can deliver external nutrients but at a low carbon cost. Both E-Strain and Unknown 1 form weak associations in that the mantle is only partially developed (see Chapter 2) and this may indicate a facultative relationship. E-Strain fungi are known to be easily culturable on agar (Danielson, 1982; Wilcox *et al.*, 1983) suggesting that they may have some saprotrophic capabilities. There has been little work on the functional characteristics of E-strain fungi in association with plants. However it would seem that under certain conditions, inoculation with E-strain fungi can improve survival rates and shoot growth compared to uninoculated controls suggesting mutually beneficial associations with plants (Yu *et al.*, 2001). Thus if these fungi are exercising a degree of nutritional versatility by deriving carbon from soil organic matter as well as from the *Cistus* seedlings the carbon cost to the plant in exchange for nutrients will be relatively low.

The virtual absence of basidiomycete morphotypes from the upper portion of root systems is likely to be due to the removal of the litter layer by direct consumption by fire and loss of inoculum in the upper few centimeters of the soil through lethal heating. What then are the inoculum sources for E-Strain and Unknown 1?

E-strain fungi are known to form thick-walled chlamydospores that probably persist in mature forest systems for several years (Danielson, 1982; Wilcox *et al.*, 1974). These may be heat resistant and remain viable after wildfires (Visser, 1995). Many other post-fire ascomycetes produce propagules that are stimulated to germinate by short exposures to temperatures of 50 °C (El-Abyad & Webster, 1968; Wicklow & Zak, 1979; Zak & Wicklow, 1980). The heat at the soil surface during a wildfire would be considerably higher than 50 °C but soon dissipates due to the insulating properties of soils (see Chapter 1). It is also the case that in Mediterranean pine forest soils there is little development of an organic horizon. The very dry litter lies almost directly on top of hard mineral soil. As the vegetation and litter are highly flammable they burn intensely and therefore rapidly and forest fires probably move quickly through stands. Thus soil heating may be short-lived and this may limit the depth to which it penetrates. Thus heat resistant propagules of fungi present below the zone of lethal heat may be stimulated to germinate by the heat of a fire. These fungi could then proliferate in the uppermost soil layers with little competition from the basidiomycete ectomycorrhizal fungi that normally dominate the litter layer of the undisturbed forest.

While E-Strain, Unknown 1 and *Tuber* morphotypes were all relatively common throughout the forest, i.e., occurring at both shrub and open microsites, some fungi were found only at shrub microsites. Most notably, all but one of the morphotypes identified as basidiomycetes were found only at shrub microsites. All but one of these was found predominantly in the lower soil layers. These fungi appear to be colonising from sources of inoculum that are only found below the soil surface around shrubs. It is unclear at present what these sources are. Any explanation must account for the fact that these basidiomycetes are occurring beneath non-ectomycorrhizal as well as ectomycorrhizal shrubs. It may be due to accumulation of basidiospore-rich soil around resprouting stems (Amaranthus & Trappe, 1993). However the maintenance of the vertically stratified distribution of the different groups of fungi beneath shrubs suggests that this is unlikely to be the whole story. Colonization may be from spores or mycelium that survived the passage of fire beneath the soil surface. Alternatively, it may be from mycelium attached to living roots of ectomycorrhizal shrubs such as *Quercus coccifera*. If this were the case, the occurrence of these fungi under non-ectomycorrhizal shrubs like *Pistacia lentiscus* and *Phyllirea latifolia* could be explained by foraging of *Quercus* roots there. Capture of nutrients from the litter of non-ectomycorrhizal species by locating ectomycorrhizal roots under their canopies would be advantageous to *Quercus* individuals.

Further studies are required to ascertain the likely sources of inoculum for the morphotypes recorded in the present study and thus shed further light on the ecology of the early post-fire colonization of *Cistus* seedlings.

# Chapter 4 – A greenhouse bioassay to determine inoculum potential of ectomycorrhizal fungi colonizing *Cistus creticus* L. seedlings from resistant propagules after wildfires.

## 4.1 Introduction

In Chapter 3, the analysis of ectomycorrhizal colonization of naturally establishing *Cistus creticus* seedlings revealed that this species has a broad receptivity to ectomycorrhizal fungi (*sensu* Molina *et al.* (1992)) meaning that it can form associations with a large number of different fungal taxa. However, there appears to be some variation in the spatial distribution of the different fungi involved in the post-fire forest soils. It was hypothesised that this was related to the dominant form of the inoculum from which the different fungi involved colonize new seedlings. Fungi found in sub-surface soil layers next to resprouting shrubs were thought to be colonizing from intact, living mycelial networks while fungi found throughout the forest and dominant in the upper few centimeters of the soil were thought to be colonizing from spores. This chapter reports the results of a greenhouse bioassay that was designed to elucidate the most likely form of inoculum of the different ectomycorrhizal fungi in post-fire forest soils and to assess their potential for colonization.

Traditionally, greenhouse bioassays have been conducted by mixing sample soils in serial dilution with sterilised soil to assess most probable number (MPN) of spores. More recently it has been recognised that spores are not the only form of colonizing propagules of ectomycorrhizal fungi and that the interpretation of studies using MPN methods is therefore limited (Brundrett & Abbott, 1995). Bioassays are now commonly used to assess treatment or site effects on the inoculum potential of root-colonizing fungi in soils. Inoculum potential of ectomycorrhizal fungi has been defined as the capacity of their propagules to form associations and can be measured as the rate of colonization of host roots (Brundrett & Abbott, 1995). This definition encompasses all propagules within the soil, including spores, mycelium and colonized root fragments and recognizes that the less disturbance there is to the soil during sampling, the greater the chance of assessing colonization from all propagule types.

However, even with the use of intact soil cores, greenhouse bioassays are still biased towards those fungi that can readily colonise from propagules that are isolated from host plants. Within the context of the 'late-stage/early stage' concept of ectomycorrhizal succession introduced in Chapter 1, early-stage, pioneering fungi will dominate in greenhouse bioassays because they are more highly competitive than late-stage fungi when isolated from host

plants. In the field, late-stage fungi, attached to their hosts, are more competitive and therefore dominant. Thus greenhouse bioassays are at their most informative when compared to studies of naturally establishing seedlings at the same sites. Fungi found colonizing naturally establishing seedlings but not greenhouse bioassay plants are likely to be 'late-stage' type fungi that colonize from extra-radical mycelium and/or rhizomorphs that are attached to intact ectomycorrhizas.

This comparative approach has been used to good effect in studies of pre- and post-fire ectomycorrhizal fungal communities in North America. Such studies conducted in mature *Pinus muricata* forests have revealed a striking lack of similarity between the fungal community colonising roots in the mature forest and that colonising roots of bioassay seedlings grown in the same soil samples (Taylor & Bruns, 1999). Although both communities were dominated by *Tomentella sublilacina*, the associated dominants in the mature forest roots were species of *Russula, Lactarius* and *Amanita* and in the bioassay seedlings, species of *Rhizopogon*, *Tuber* and *Wilcoxina*. This indicates that these latter species were colonizing primarily from isolated propagules and it has been proposed that these fungi form soil spore banks that are analagous to plant seed banks (Taylor & Bruns, 1999). After a wildfire had burned this same study site, the fungi dominating naturally establishing seedlings and seedlings grown in greenhouse bioassays of the burned forest soils were similar to each other and closely resembled those dominating the spore bank community from the previous study (Baar *et al.*, 1999). Although reduced in abundance, the pre-fire dominants were also present on root systems of the naturally establishing seedlings immediately after the fire (Horton *et al.*, 1998) but, with the exception of *Tomentella sublilacina*, not the bioassay seedlings (Baar *et al.*, 1999).

This chapter describes a greenhouse bioassay using *Cistus creticus* as bait plants in soil cores taken from the same sites where the ectomycorrhizal fungal community colonizing naturally established seedlings has been described (Chapter 3). The main objective of the bioassay was to characterise which of those fungi could have colonized from resistant propagules and to assess their presence in the unburned forest. A second objective was to assess the effect of proximity to resprouting shrubs on the inoculum potential of those morphotypes colonizing from resistant propagules.

## 4.2 Methods

### 4.2.1 Study sites

Soil was collected from the same three burned forest sites described in Chapter 3 (see Section 3.2.1). At each of the sites, a matching unburned area of forest of estimated similar composition and physiognomy to the burned site was selected. The burned and unburned sites in each of these three areas are indicated by the postscripts "B" and "UB" respectively (e.g., KOR_B = burned and KOR_UB = unburned).

The characteristics of the burned sites have already been described elsewhere (Chapter 3, Section 3.2.1). The characteristics of the unburned sites were recorded in the same way except that percentage cover of rocks was not recorded in the unburned sites due to the confounding influence of litter cover. Understorey species composition was also recorded at both burned and unburned sites using the DAFOR scale in the same way as previously described.

The site characteristics and understorey species composition for all of the sites are presented for comparative purposes in Tables 4.1 and 4.2 respectively.

### 4.2.2 Sampling

#### *a) Occurrence of fungal fruitbodies*

During the sampling period and during further site visits in December any fruitbodies encountered were recorded and collected. These were returned to the University of Athens where they were identified to at least genus by Zapi Gonou-Zagou of the University Mycology Department. Specimens were deposited in the mycological herbarium at the University of Athens.

#### *b) Bioassay samples*

Sampling was carried out during the period 5/11/99 to 13/11/99. At each of the sites three resprouting individuals of each of an ectomycorrhizal shrub ["EM-shrub"] (*Quercus coccifera*) and a non-ectomycorrhizal shrub ["Non-EM shrub"] (either *Pistacia lentiscus* or *Phyllirea latifolia*) were randomly selected from a pool of 10 selected shrubs of each type. EM-shrub and Non-EM-shrub are henceforth referred to as 'shrub type'. At each selected shrub two samples of approximately 260 $cm^3$ of the top 10 cm of soil were collected with a trowel. One sample was taken from the shrub canopy zone, within 30 cm of the base of resprouting stems and one from adjacent open areas greater than one metre from any resprouting stems. Sample position relative to individual shrubs (i.e., 'canopy' and 'open') is henceforth referred to as 'location'.

Table 4.1 Characteristics of burned and unburned sites at Longos (LON), Agios Konstandinos (AgK) and Korfos (KOR). Figures indicate mean per site (± se).

| | LON_B Burned | LON_UB Unburned | AgK_B Burned | AgK_UB Unburned | KOR_B Burned | KOR_UB Unburned |
|---|---|---|---|---|---|---|
| n | 9 | 9 | 8 | 8 | 6 | 6 |
| Slope | Steep (>30°) | Steep (> 30°) | Steep (>30°) | Steep (>30°) | Moderate (~20°) | Moderate (~20°) |
| Aspect | SE | E | N | N | S | S |
| % cover of rocks on surface | 5.3 (±0.7) | - | 24.4 (±2.9) | - | 25.3 (±0.9) | - |
| Stand density (No. trees $ha^{-1}$) | 385.5 (±54.7) | 261.7 (±20.4) | 147.2 (±18.0) | 143.2 (±18.0) | 275.9 (±28.1) | 360.8 (±33.6) |
| DBH (cm) | 21.7 (±0.5) | 27.6 (±1.4) | 22.6 (±1.4) | 33.9 (±2.3) | 21.3 (±0.7) | 19.1 (±1.4) |

Table 4.2 Occurrence of understorey species at sites sampled in Bioassay 3 (D=Dominant; A=Abundant; F=Frequent; O=Occasional; R=Rare; + = Present; - = Absent. See text for definitions).

| | AgK_B | AgK_UB | KOR_B | KOR_UB | LON_B | LON-UB |
|---|---|---|---|---|---|---|
| *Arbutus andrachne* | - | - | - | - | R | - |
| *Arbutus unedo* | A | A | - | - | A | A |
| *Asparagus acutifolius* | - | O | - | - | - | - |
| *Bupleurum fruticosum* | - | - | - | - | - | F |
| *Calycotome villosa* | - | - | - | - | - | F |
| *Cistus creticus* | - | - | - | D | - | - |
| *Cistus salvifolius* | - | - | - | - | - | F |
| *Clematis vitalba* | - | - | - | - | F | - |
| *Cyclamen sp.* | - | - | R | R | A | R |
| *Ephedra sp.* | - | - | - | - | - | R |
| *Olea europaea* | F | - | - | - | - | - |
| *Phyllirea latifolia* | F | A | D | A | - | A |
| *Pistacia lentiscus* | F | F | A | A | A | A |
| *Pistacia terebinthus* | - | O | - | - | - | - |
| *Quercus coccifera* | A | A | A | D | F | A |
| *Smilax aspera* | A | A | - | - | - | - |

To minimise disturbance soil samples were carefully wrapped in aluminium foil and packed into open-ended PVC cylinders that were then sealed in plastic bags and placed in an insulated coolbox. Instruments were cleaned between each sample. Samples were returned to the laboratory within two days of collection and stored at 4 °C.

### *c) Soil nutrient samples*

In addition to the soil cylinders collected for the bioassay, two further soil samples were collected at each shrub location for chemical analysis. These were collected to a depth of 10 cm from positions immediately adjacent to the first two samples and were placed in separate labelled plastic bags.

These soil samples were placed in foil trays and air dried at room temperature for 7 days. They were then sieved to 4 mm to remove large stones, ground in a large pestle and mortar

to break up aggregates, sieved to 2 mm and stored at room temperature in self-sealing plastic bags. Samples were returned to the UK and stored at 4 °C until analysed.

Soil chemical analyses were carried out by the Scottish Agricultural College Analytical Services Department. The samples were analysed for pH (calcium chloride suspension), phosphorus (Olsen's method), potassium and magnesium (extraction with modified Morgan's solution).

### 4.2.3 Seed germination

Seed capsules of *Cistus creticus* were collected from the hillside just outside of the University grounds on 13/9/99 and stored at 4 °C until use.

On 23/11/99 *Cistus creticus* seed was scarified by rolling between two sheets of fine grade sand paper and surface sterilised by immersion in 30% hydrogen peroxide in glass vials placed on a horizontal agitator for 10 minutes. The seed was then transferred to a sterile metal tea strainer and rinsed with sterile de-ionised water. The seed was surface dried in an oven at 50 °C for 10 minutes and then transferred onto moist filter paper in sterile Petri dishes (15 cm) in batches of 100. Seed was germinated under continuous darkness at 15 °C in a growth cabinet. After six days just over 50% of the 800 seeds had germinated.

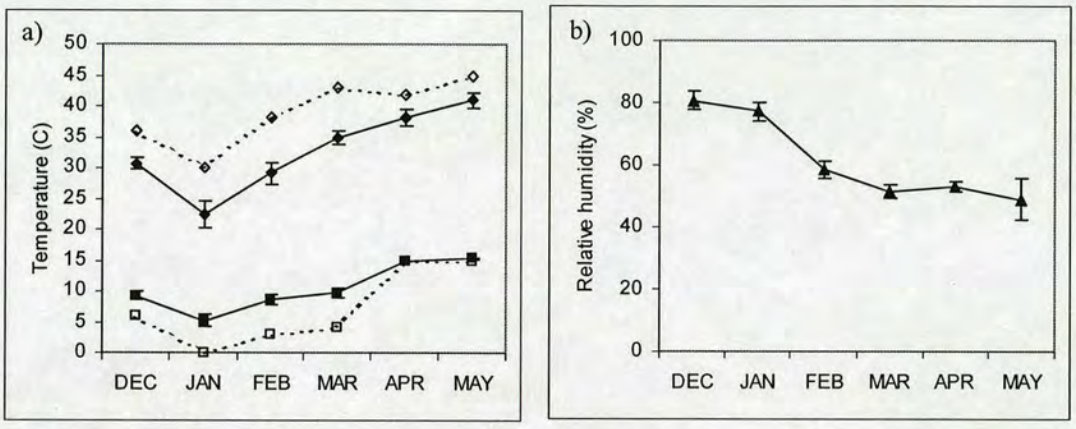
### 4.2.4 Experimental set-up

The PVC cylinders containing the soil samples were placed in individual drip trays and arranged in a completely randomized design in a purpose-built greenhouse. The aluminium wrapping the soil cores was pierced at the bottom for drainage and the top covering of aluminium was removed.

On 1/12/99 the soil cylinders were planted with the pre-germinated seeds of *Cistus creticus* and 30 ml of de-ionised water was applied. All seedlings had fully expanded cotyledons at planting. The healthiest looking seedlings were selected and then randomly assigned to each of the 72 soil cylinders. During the first week mortalities were replaced as required. The seedlings were watered as required for the first month and then given 20 ml de-ionised water two times per week thereafter.

During the course of the bioassay the minimum and maximum daily temperature were recorded with a Min/Max thermometer and the relative humidity was recorded with a wet/dry thermometer. The average monthly minimum and maximum temperatures for the months inside the greenhouse are shown in Figure 4.1

Figure 4.1 Environmental conditions in the greenhouse during the bioassay. a) Temperature. Solid lines are mean (± se) monthly minimum (■) and maximum (♦) temperature. Broken lines are absolute minimum (□) and maximum (◇) temperatures recorded in each month. b) Relative humidity. Values for relative humidity are monthly means (± se).




### 4.2.5 Harvest

The experiment was ended on 8/4/00, 120 days after initial planting. Out of the 72 plants at the start of the experiment, 69 survived. Shoots were removed and oven dried at 80 °C for five days. Dried stem and leaf fractions were weighed separately. The soil cylinders containing the root systems were stored at 4 °C until further processed.

The soil cylinders were immersed in water in treatment groups for 2 days prior to root recovery. The soaked soil cylinders were then carefully removed from the aluminium foil and the root systems were washed and recovered over a series of sieves (4.75 mm, 1 mm, 0.5 mm) using running tap water. Each soil sample was passed through the sieve series twice and detached root fragments were washed into a white basin for recovery. The root systems and root fragments were placed in Petri dishes (15 cm) and individually cleaned of soil and organic debris using forceps under a dissecting microscope. The fresh weight of each root system was recorded after careful drying with absorbent paper. The root systems were then stored at 4 °C in 2% Glutaraldehyde in 30 ml plastic vials.

### 4.2.6 Mycorrhizal assessment

*a) Short roots*

In November 2001, the root systems were assessed for mycorrhizal colonisation. Small root systems were examined intact in Petri dishes in water under a dissecting microscope. All root tips were recorded as non-mycorrhizal or according to ectomycorrhizal morphotype.

Larger root systems were cut into fragments of approximately 1 cm length and randomly dispersed in water in a clear perspex tray measuring 17 x 11 cm marked with a grid of 100 squares. All root tips were recorded as non-mycorrhizal or according to ectomycorrhizal morphotype by systematically scanning each grid square.

Three measures of colonization were used to assess the behaviour of individual ectomycorrhizal fungal species: percentage of total root tips colonized, percentage of ectomycorrhizal root tips and percentage of seedlings colonized. The first is a measure of individual inoculum potential, which is related to propagule numbers and also probably nutritional status of the fungus. The second is a measure of relative abundance that also relates to numbers of propagules but may also reflect competitive ability. The third is a measure of frequency and relates to distribution of inoculum.

***b) Long roots***

For clearing and staining of roots to examine internal colonization, entire root systems were cut into fragments of approximately 1 cm length and placed in labelled modified syringes in water. For the larger root systems already cut into fragments and dispersed in the perspex tray, root fragments from 10 randomly selected grid squares were removed to labelled modified syringes in water. The clearing and staining procedure is described in Chapter 2.

For quantification of long root colonization, 10 of the 1 cm fragments were mounted in PVLG under cover slips on microscope slides. Fragments were orientated parallel to the long axis of the slides. Samples of more than 10 fragments were suspended in water in 5 cm Petri dishes, placed over graph paper and fragments lying on or closest to the centre of 10 randomly selected grid squares were selected.

The mounted root fragments were scanned under a compound microscope at 1 mm intervals at ×200 magnification in one plane. Root colonization was recorded at each intersection between root and eye-piece crosshair graticule (McGonigle *et al.*, 1990). At each intersection, root colonization was scored as one of four classes representing increasing fungal loading: 1) no colonization; 2) running hyphae only; 3) running hyphae + Hartig net; and 4) mantle + Hartig net (Table 4.3). Short roots emanating from mounted long root fragments were not scored. The number of intersections of each class was expressed as a

percentage of the total number of intersections. The combination of all classes is referred to as percentage of root length colonized.

### 4.2.7 Statistical analysis

The data were analysed as a split-plot design. Three sites were selected, each with a burned and unburned treatment. One block was established in each of the burned and unburned sites. The blocks were each split into six plots (3 × EM and 3 × non-EM shrubs) that were each sampled at two locations (canopy and open). The effect of site and fire were analysed within the block stratum. The effect of shrub type and its interaction with fire were analysed within the block × plot stratum. The effect of location and its interactions with fire and shrub type were analysed within the block × plot × location stratum. The ANOVA structure and degrees of freedom for the parameters analysed are shown in Table 4.4.

Analyses were carried out on the untransformed data and the residuals checked for normality (Anderson-Darling test). Data with non-normal residuals were transformed (Log(x+1) or arcsine($\sqrt{x}/100$)), re-analysed and the residuals checked again. Homogeneity of variance was checked with Bartlett's test.

Relationships between seedling growth parameters and root colonization were tested by calculating Pearson's product moment correlation coefficients.

## 4.3 Results

### 4.3.1 Fruitbodies

Few fungi were found fruiting during the sampling period. Those found in the unburned sites were mostly ectomycorrhizal species of Basidiomycetes within the orders Agaricales (*Amanita* sp., *Hygrophorus* sp., *Hebeloma* sp.), Boletales (*Suillus* sp., *Boletus* sp., *Xerocomus* sp.) and Russulales (*Russula* sp.) (Table 4.5). The number of fruitbodies observed of each of these taxa was low.

The burned sites were completely dominated by *Anthracobia* cf. *macrocystis* that was present in great numbers in both November and December (Table 4.5). In addition to these only two specimens of known ectomycorrhizal genera were found in the burned sites. These were a species of *Lactarius* and one of *Inocybe*. However the specimens were poor and their exact identity could not be determined.

Table 4.3 Summary of criteria for the categorization of long root colonization of *Cistus creticus* seedlings grown in forest soils in a greenhouse bioassay. Scale Bars = 50 μm.

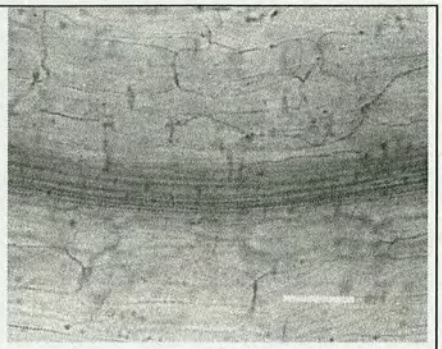
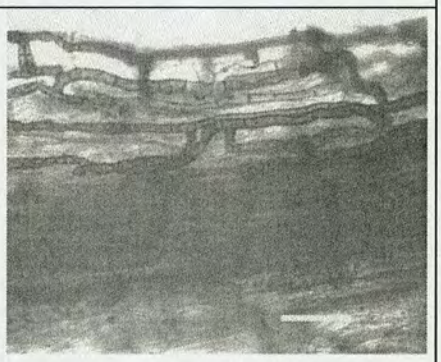
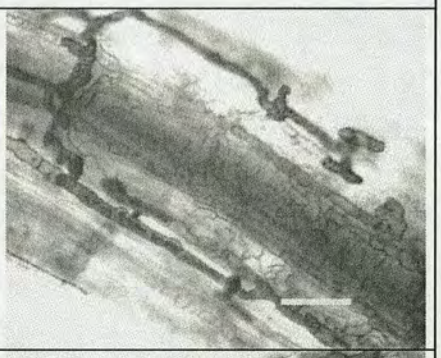
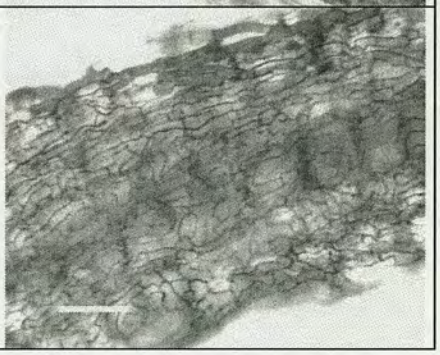
| | | |
|---|---|---|
| 1. No colonization | |  |
| 2. Running hyphae only | One or more hyphae running externally along the root surface often along the furrow between root epidermal cells. Or running internally between the root cortical cells. |  |
| 3. Running hyphae + Hartig net | In addition to the running hyphae, one or more cortical cells along the line of intersection associated with densely ramified hyphae forming palmetti on the cell surface. |  |
| 4. Mantle + Hartig net | In addition to running hyphae and Hartig net, many external runner hyphae coalesced on the root surface to form a rudimentary mantle |  |

Table 4.4 ANOVA structure and degrees of freedom (df) for parameters analysed. A – Percentage of root tips colonized, percentage of root length colonized, all seedling growth parameters. B – Percentage of root tips colonized by morphotype Unknown 1. C – Soil chemistry parameters

| Source | df | | |
|---|---|---|---|
| | A | B | C |
| **Block stratum** | | | |
| Site | 2 | 2 | 2 |
| Fire | 1 | 1 | 1 |
| Residual | 2 | 2 | 2 |
| **Block.Plot stratum** | | | |
| Shrub | 1 | 1 | 1 |
| Fire.shrub | 1 | 1 | 1 |
| Residual | 28 | 28 | 4 |
| **Block.Plot.Location stratum** | | | |
| Location | 1 | 1 | 1 |
| Location.Fire | 1 | 1 | 1 |
| Location.Shrub | 1 | 1 | 1 |
| Location.Fire.Shrub | 1 | 1 | 1 |
| Residual | 29 | 25 | 8 |
| Total | 68 | 64 | 23 |

Table 4.5 List of fruitbodies observed in November and December 1999 in burned and unburned forest sites in Central Greece. The fires at the burned sites occurred in August 1999.

| Site | Identification |
|---|---|
| Unburned | *Suillus* sp., *Boletus* sp., *Xerocomus* sp., *Clitocybe* sp., *Leucopaxillus amarus*, *Hygrophorus* sp., *Hebeloma* sp., *Russula* sp., *Hydnum repandum*, *Amanita ovoidea*, *Hygrocybe* sp. |
| Burned | *Anthracobia* sp., *Coprinus* sp., ?*Omphalotus* sp.(immature specimen), ?*Lactarius* sp. (poor specimen), ?*Inocybe* sp. (poor specimen) |

### 4.3.2 Bioassay soil inoculum potential

The overall mean percentage of root tips colonized was 23.1% ±1.73 s.e. A higher percentage of root tips were colonized in the unburned soil compared to the burned soil ($F_{1,2}$ = 18.51, $P$ = 0.05) (Figure 4.2a)

The overall percentage of lateral root length colonized was 32.5% ±2.67 s.e. There was a significant interaction between fire and shrub type for the percentage of root length colonized ($F_{1,28}$ = 4.79, $P$ = 0.037). Burning slightly increased the percentage of root length colonized at all locations except open areas associated with non-EM shrubs where it decreased quite dramatically (Figure 4.2b).

Proportions of root length colonized by the fungal load classes are shown in Figure 4.3. Fungal load was partitioned as 10.7% ±1.0 running hyphae, 19.5% ± 2.0 running hyphae with Hartig net, 2.2% ±0.4 mantle with Hartig net. Thus 21.7% ±2.7 of the lateral roots had some Hartig net development. Partitioning did not vary significantly with burning or location.

### 4.3.3 Morphotypes

Only six morphotypes were recorded from the bioassay seedlings. Four of these (Unknown 1, *Tuber* sp. 1, Unknown 2, *Inocybe* sp. 1) were also recorded on the naturally established seedlings at the same sites (Chapter 3) and two (*Genea*-like, Unknown 12) were additional taxa. A greater number of morphotypes was recorded in the unburned soils but their frequency was so low that patterns of occurrence with respect to location are not discernible. Though present on few

Figure 4.2 Effect of fire, shrub type and proximity to shrub on bioassay soil inoculum potential quantified as a) percentage of root tips colonized and b) percentage of root length colonized. Error bars = standard error. White bars = unburned, black bars = burned.

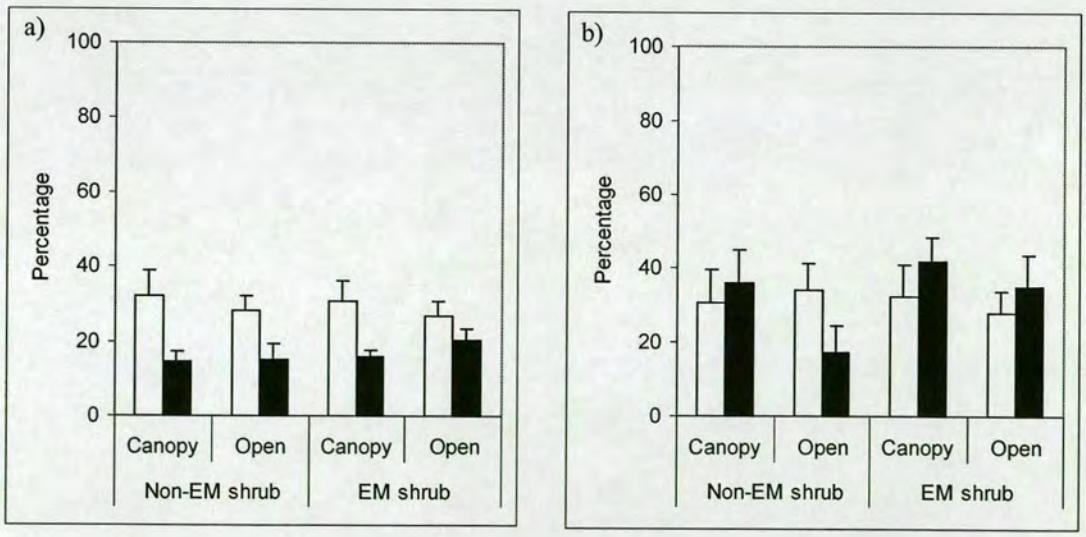
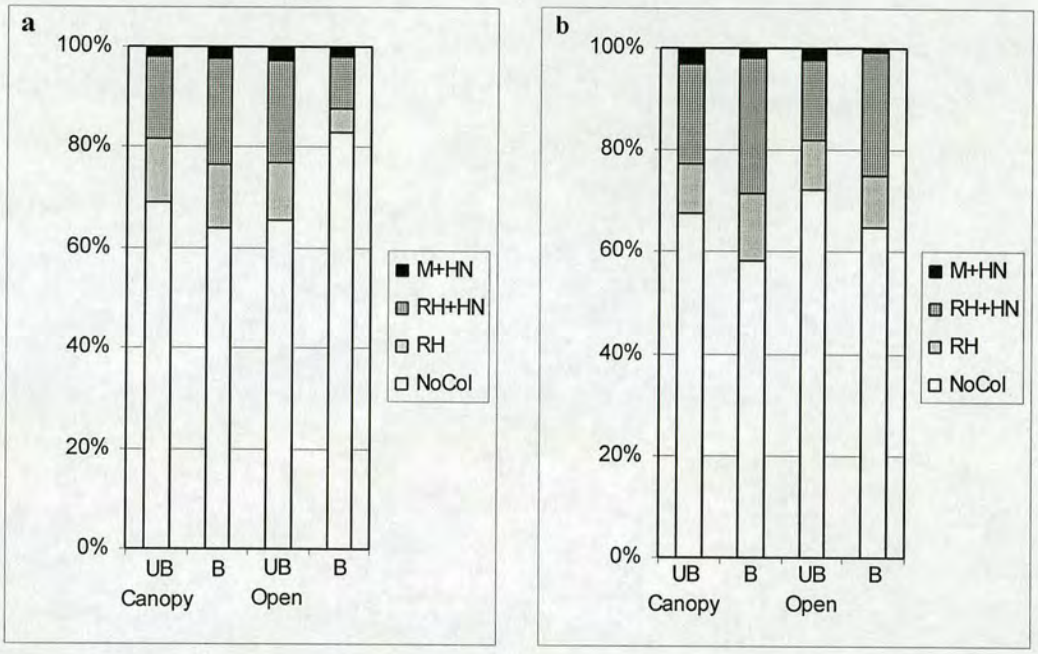


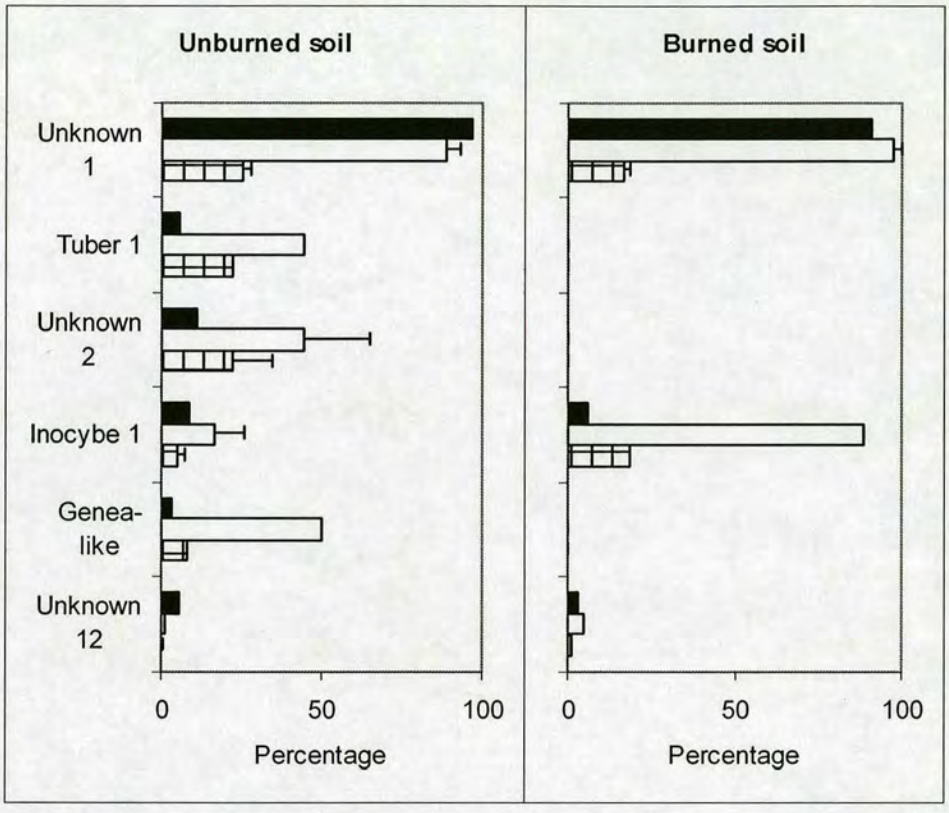
Figure 4.3 Proportions of root length colonized attributed to four fungal load classes at a) non-EM and b) EM shrub and associated open locations. Colonization categories are: NoCol, no colonization; RH, running hyphae only; RH+HN, running hyphae + Hartig net; M+HN, mantle + Hartig net.




seedlings, where they did occur, morphotypes *Tuber* sp. 1, Unknown 2, *Inocybe* sp. 1, and *Genea*-like were well represented in terms of their relative abundance and percentage colonization (Figure 4.4).

By far the most abundant and frequent morphotype was Unknown 1 which occurred in all treatment groups and colonized 65 of the 69 seedlings that were alive at the end of the experiment (Figure 4.4). The complete dominance of this morphotype is demonstrated by the fact that the next most frequent morphotype, *Inocybe* sp. 1, colonized only five seedlings in total. The inoculum potential of Unknown 1, as measured by percentage of root tips colonized was unaffected by location but was greater in the unburned soils (25.7% ± 2.38 s.e) than in the burned soils (16.9% ± 1.5 s.e) ($F_{1,2} = 16.65$, $P = 0.055$). Due to the general absence of other morphotypes, the relative abundance of Unknown 1 was unvaryingly high. Across all seedlings where present, Unknown 1 occurred with an abundance relative to other morphotypes of 93.0% ± 2.4 s.e.

Figure 4.4 Effect of burning on frequency (black bars), relative abundance (white bars) and percentage colonization (hatched bars) of morphotypes colonizing bioassay seedlings. Values for relative abundance (percentage of colonized root tips) and percentage colonization (percentage of total root tips) are means. Error bars are standard error where n > 2.



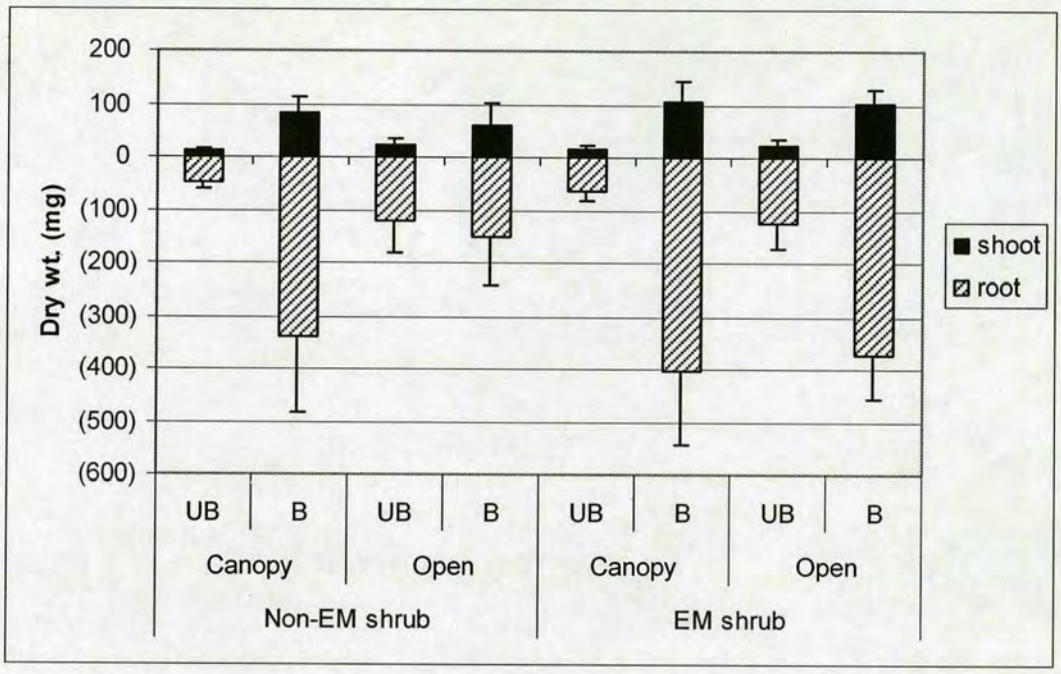

Based on morphological characteristics of the hyphae, colonization of lateral roots recorded as percentage of root length colonized in section 4.3.2 appeared to be by the same fungus associated with morphotype Unknown 1 (see Chapter 2, section 2.5.1b for description of long root colonization by this fungus).

### 4.3.4 Seedling growth

Shoot and root dry weight showed a positive response to burning that varied according to shrub type (Fire x Shrub interaction: $F_{1,28} = 6.1$, $P = 0.017$; $F_{1,28} = 6.5$, $P = 0.020$). The response was stronger around *Quercus coccifera* shrubs compared to non-ectomycorrhizal shrubs (Figure 4.5) The differential in response between canopy and open locations appeared slightly larger at non-ectomycorrhizal shrubs compared to *Quercus coccifera* shrubs where there was little difference though all interactions with the Location term were insignificant.

The error associated with growth response was larger in seedlings growing in burned soils compared to unburned soils.

Figure 4.5 Effect of fire, shrub type and proximity to shrub on shoot and root dry weight (g) of *Cistus creticus* seedlings grown in forest soils in a greenhouse bioassay. Error bars = standard error.




There were also significant interactions between fire and shrub type for stem basal diameter, stem height, stem and leaf dry weight (Table 4.6). Variation in all of these growth parameters followed the same pattern as that presented for shoot and root dry weight in Figure 4.6. There was no significant response of number of leaves though this parameter followed the same pattern of variation as the other parameters (Table 4.6). Root:shoot ratio was unaffected by any of the treatments and had an overall mean value of 3.97 ± 0.16 s.e.

There were no significant correlations between percentage of root tips colonized and any of the growth parameters. All growth parameters were significantly positively correlated with percentage of root length colonized. However, most correlations were weak with the maximum level of association shown with root dry weight ($r = 0.528$, $p < 0.001$, $n = 69$). The strongest correlations between root length colonization and seedling growth were observed for fungal load class 3 (running hyphae + Hartig net) (Figure 4.6).

Table 4.6 Effect of fire, shrub type and proximity to shrub on the growth of bioassay seedlings. Values presented as means (± se).

| | Non-EM | | | | EM | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Canopy | | Open | | Canopy | | Open | | Significant interactions* |
| | B | UB | B | UB | B | UB | B | UB | (all FIRE xSHRUB) |
| n | 9 | 8 | 7 | 9 | 9 | 9 | 9 | 9 | |
| Stem basal diameter (mm) | 0.64 ±0.15 | 0.32 ±0.05 | 0.48 ±0.17 | 0.43 ±0.08 | 0.78 ±0.14 | 0.41 ±0.05 | 0.84 ±0.11 | 0.39 ±0.06 | p=0.019 |
| Stem height (mm) | 37.8 ±5.6 | 24.3 ±2.5 | 33.3 ±5.5 | 26.2 ±2.8 | 45.6 ±4.9 | 24.7 ±2.6 | 44.4 ±6.4 | 23.0 ±2.8 | p=0.026 |
| Stem dry weight (g) | 0.016 ±0.006 | 0.003 ±0.001 | 0.012 ±0.008 | 0.005 ±0.002 | 0.022 ±0.007 | 0.004 ±0.001 | 0.022 ±0.007 | 0.004 ±0.001 | p=0.010 |
| Leaf dry weight (g) | 0.067 ±0.027 | 0.009 ±0.002 | 0.047 ±0.035 | 0.019 ±0.009 | 0.082 ±0.033 | 0.013 ±0.004 | 0.080 ±0.021 | 0.019 ±0.008 | p=0.021 |
| Number of leaves | 20.9 ±5.8 | 8.5 ±0.7 | 13.7 ±5.1 | 9.6 ±0.7 | 21.8 ±6.6 | 9.8 ±0.8 | 22.9 ±5.3 | 12.0 ±3.3 | n.s |
| Root:shoot ratio | 3.6 ±0.5 | 3.9 ±0.1 | 3.5 ±0.5 | 4.1 ±0.5 | 4.1 ±0.4 | 3.8 ±0.2 | 4.0 ±0.5 | 4.7 ±0.5 | n.s |

*ANOVA with 1 and 28 degrees of freedom. All parameters were log transformed prior to analysis of variance.

### 4.3.5 Soil chemistry

There were no significant treatment effects on soil pH or magnesium. There was a significant effect of proximity to shrub (location) on soil phosphorus ($F_{1,8} = 6.5, P = 0.034$) (Figure 4.7). In burned soils there was greater P enrichment close to the shrubs compared to adjacent open areas. Levels of soil potassium were enriched significantly by burning ($F_{1,2} = 42.8$, $P = 0.023$) (Figure 4.7). Potassium was the only nutrient varying significantly between sites, being slightly higher at KOR than at the other two sites ($F_{2,2} = 82.8, P = 0.012$).

## 4.4 Discussion

Figure 4.6 Correlations between percentage of root length colonization categorized as fungal load class 3 (running hyphae + Hartig net) and a) shoot dry weight, b) root dry weight, c) stem basal diameter, d) stem height, e) number of leaves.

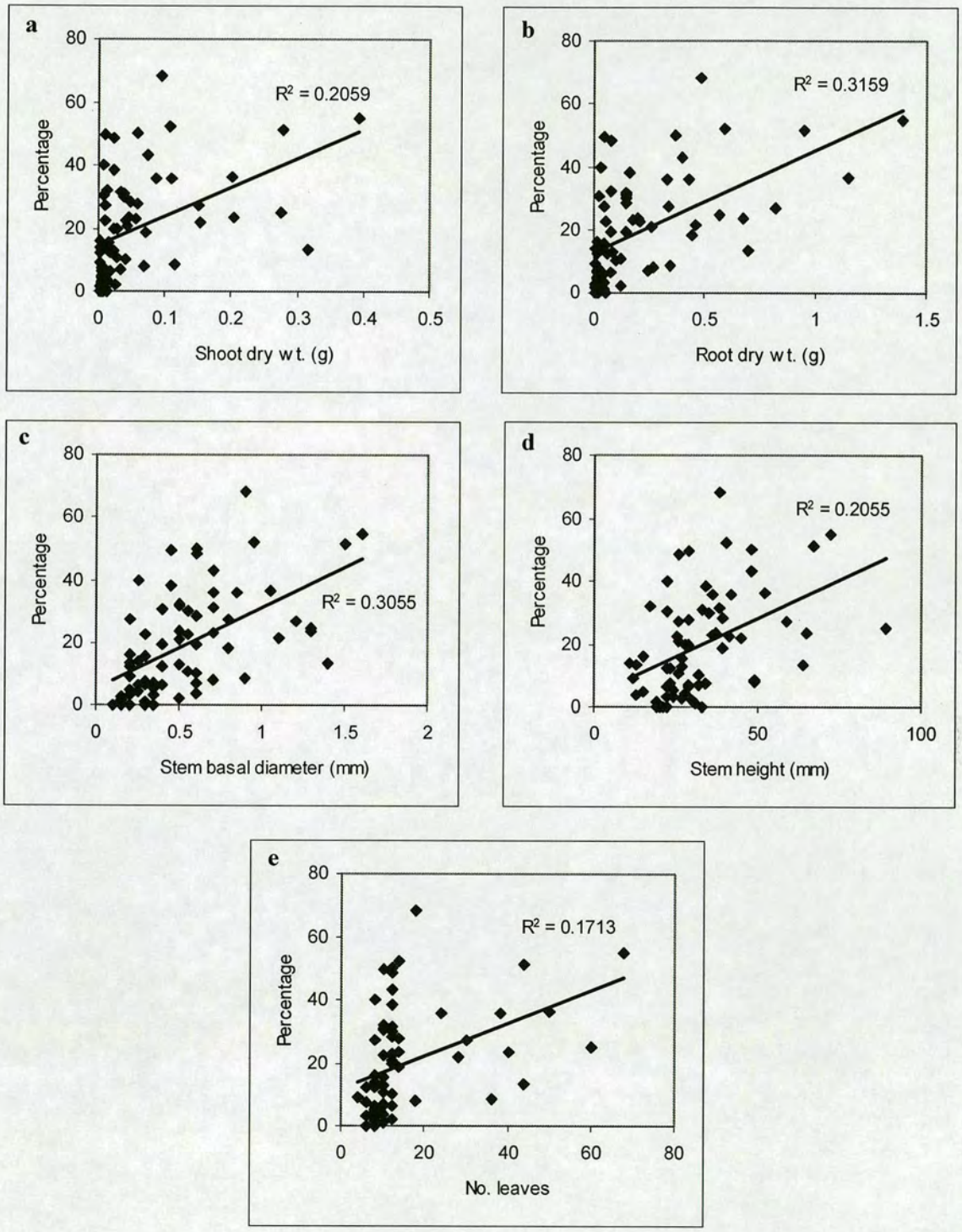
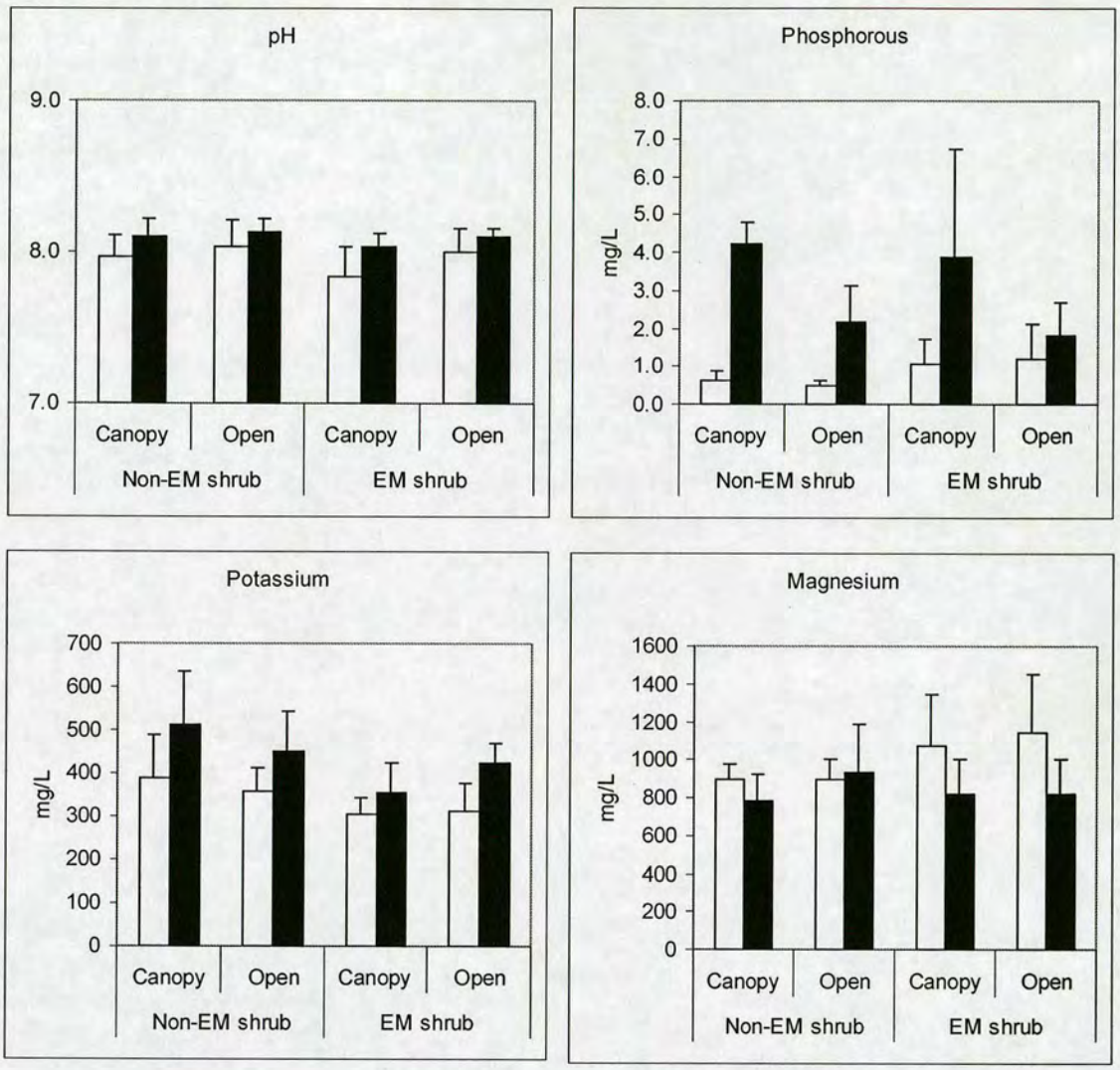


Figure 4.7 Effect of fire, shrub type and proximity to shrub on soil pH, phosphorus, potassium and magnesium. Error bars = standard error. White bars = unburned soil, black bars = burned soil.




Very few morphotypes were recorded from the bioassay seedlings: a mere six types compared with 23 found on naturally establishing seedlings at the same sites (Chapter 3). A previous bioassay of burned Mediterranean soils also resulted in low numbers of mycorrhizal morphotypes. Torres & Honrubia (1997) recorded only seven morphotypes in bioassays conducted with *Pinus halepensis* seedlings grown for six months in soils collected from two burned and one unburned forest sites in Spain up to two years after a wildfire. By contrast, Taylor & Bruns (1999) recovered 21 morphotypes from bioassays of soil from cores taken from an unburned *Pinus muricata* stand in California, USA. The same cores had yielded 20 morphotypes on the excised roots of mature plants though there was little overlap between

the two communities. After a wildfire at the same forest site, Baar *et al.* (1999) recorded eight morphotypes among bioassay seedlings compared to seven types among naturally established seedlings with four types shared between the two regimes. Elsewhere, *Pseudotsuga menziesii* seedlings grown in potted soils from forest sites in southwestern Oregon that had been clear-cut and broadcast burned 5 years previously, formed only five ectomycorrhizal morphotypes (Borchers & Perry, 1990). It is difficult to place the current findings in context by making direct comparisons between studies due to the differences in host species and experimental conditions used in the bioassays. It is also true that few studies have made direct comparisons of morphotype diversity between naturally establishing and bioassay seedlings such that many bioassay trials can not themselves, be interpreted in context. However it is likely that the present figure is at the lower end of the spectrum.

The discrepancy in numbers of morphotypes recorded between naturally established seedlings and bioassay seedlings in the present study could be explained by the earlier sampling of the bioassay soils in the November following the fire compared to the assessment of naturally established seedlings which was carried out in February. The greater diversity of morphotypes found in February may have been the result of spore inputs from fruitbodies appearing between November and February. However, apart from the ubiquitous *Anthracobia* fruitbodies, very few other fungi were found at any of the burned sites during site visits in November and December or during the excavation of seedlings in February. Another possibility is that spores of other fungi may have blown in from adjacent unburned areas. However, the few ectomycorrhizal fungal genera encountered were already fruiting in the unburned forest in November while the bioassay soils were being collected and these fungi did not generally show up in the bioassay. Furthermore, the difference in ectomycorrhizal diversity between the natural and bioassay regimes cannot be explained by differences in seedling maturity as most of the bioassay seedlings were more developed by the end of the experimental period than the field seedlings. The naturally established seedlings had germinated in October and were harvested in February while the bioassay seedlings were planted at the beginning of December into soils that had been collected in November and were harvested at the beginning of April.

It is therefore reasonable to assume that few fungi colonizing naturally establishing seedlings in these post-fire forest soils are doing so from spore or fragmented mycelial inocula. Other possible sources of inocula are intact mycelium connected to new living roots of resprouting shrubs or mycelium connected to moribund roots. The latter have previously been identified as possible inoculum sources (Brundrett & Abbott, 1995; Ferrier & Alexander, 1985). It is perhaps not surprising to find that most of the fungi recovering after a wildfire do so

vegetatively from subterranean sources that survive the fire. 'Resprouting' is the very strategy that has evolved in many of the understorey shrubs of fire-prone areas. The other common strategy is that of seeding and this too appears to have a mycological counterpart in fungi like Unknown 1.

Despite the presence of five other morphotypes recorded in the bioassay the seedlings were completely dominated by morphotype Unknown 1 in both burned and unburned soils. This morphotype was the second most common type found on the naturally established seedlings after the E-strain (Chapter 3). There, along with E-strain it was found at all microsite types, occurring predominantly in the upper three centimetres of the soil. The results of the bioassay suggest that this fungus is widely dispersed in the soil of the unburned forest during the fire interval, probably as spores. Evidently, the colonizing propagules of this fungus do not require heat stimulation to germinate but are probably heat resistant to some degree given their position in the upper soil layers (Chapter 3). Within the unburned forest this fungus may be associating with the roots of mature shrubs and trees or it may persist as dormant propagules dispersed from fruitbodies during the period since the last fire. The absence of this morphotype on the roots of mature plants in unburned forest would not necessarily indicate absence of the fungus as it may only associate with seedlings, being displaced from root systems by other fungi as the plants age. As *Cistus* seedlings are rarely found in unburned forests, an appropriate method to find out would be to conduct a field bioassay, planting seedlings into burned and unburned areas.

In addition to the average of 23% of root tips colonised by Unknown 1, 32.5% of lateral root length was also colonised. 22% of this lateral root colonization included Hartig net-like formations within the cortical layers. This is an unusual pattern of colonization but appears to be an important component of this fungus. Such a pattern of colonization could be the result of some factor related to the experimental conditions. The relative humidity in the greenhouse was high during the first two months of the experiment and this could have resulted in the soil remaining wet for longer periods after watering than might be experienced under natural conditions. The significance of this pattern of colonization may only become apparent if it can be recorded systematically in nature.

The absence of E-strain morphotypes from the bioassay seedlings is curious. The E-strain group is commonly found after disturbances and has been previously recorded as a dominant morphotype in greenhouse bioassays conducted with soils from *Pinus halepensis* forests (Torres & Honrubia, 1997). It was also the most dominant fungus colonizing naturally establishing seedlings at the present study site (Chapter 3). A possible explanation may be the relatively superior competitive abilities of the fungus forming the Unknown 1

morphotype under these conditions. Again, this may be a function of the experimental conditions.

In the present study, treatment effects on bioassay soil inoculum potential appeared to vary according to which method of quantification of root colonization was used. This very much applies to the inoculum potential of Unknown 1 as this was so dominant in these soils.

In general the most marked effects were attributed to burning. The percentage of root tips colonized was greater in the unburned soils than in the burned soils. On the other hand, the percentage of root length colonized was generally greater in the burned soils. As would be expected, the data also indicate that seedling growth was enhanced in the nutrient enriched burned soils and root growth in particular was stimulated. This resulted in a larger number of root tips being produced by seedlings in the burned soils. Percentage of root tips colonized is a measure of soil inoculum potential that is most influenced by propagule numbers and dispersion within the soil because colonization is largely determined by a new root tip encountering a new fungal propagule. As the soil cores must have contained a finite number of colonizing propagules, root tip production in the nutrient-enriched burned soil is likely to have exceeded the colonizing potential of the soil. If secondary infection rates were low, this would be reflected by a lower proportion of colonized root tips in the larger root systems of the burned soils. This proposition is supported by the observation that percentage of root tips colonized by *Tuber melanosporum* was highest in pot-grown hazel plants of intermediate root system volume (Mamoun & Olivier, 1996; Olivier & Mamoun, 1994), decreasing in individuals with larger root systems.

Percentage of root length colonized is a measure of inoculum potential that is probably more influenced by the activity of the fungus after initial colonization and this appears to have been stimulated in the burned soils. This could be related to soil chemistry and/or to interactions with the host plant.

If the behaviour of Unknown 1 is related to soil nutrients we might expect to find differences between the 'canopy' and 'open' locations because nutrient inputs should be higher underneath the burned canopy of shrubs and indeed such a pattern was seen in the levels of soil phosphorus. Average percentage root length colonized in the burned soils also exhibited a trend of elevation within the canopy zone but levels of variation were high and this was not significant. Unfortunately, soil samples from the different treatment replicates were pooled within sites and so it was not possible to test for correlations between soil nutrients and colonization levels. Such analyses could provide supporting evidence for such a relationship.

An alternative, or perhaps complementary, explanation for the greater percentage of root length colonized observed in most of the burned soils compared to unburned soils is that of carbon supply. Seedling growth was greater in burned soils where levels of some nutrients were higher. Greater seedling growth results in higher levels of photoassimilate available to colonizing fungi which in turn enhances the growth and colonization potential of those fungi. Here, it was possible to examine the data for relationships between seedling growth and root colonization. Percentage of root length colonized was in fact moderately correlated with seedling size at the end of the experiment while percentage of root tips colonized was not.

It is difficult to disentangle the relative effects of soil nutrients and host carbon supply from an experiment that was designed to answer questions about the probable types of inoculum propagules that different fungi are colonizing from in the post-fire environment. Nevertheless the observations raise some interesting questions and it does highlight the importance of using a method of quantification that is appropriate to the association under investigation. These weak ectomycorrhizal associations are more akin to endomycorrhizas that are typically quantified on a percentage root length basis. As in the case of arbuscular mycorrhizas where colonization can be partitioned into arbuscules and intercellular hyphae, it is important to quantify the proportion of roots that have structures associated with nutrient exchange. In the case of ectomycorrhizas these may not necessarily be restricted to the short roots. In the present case the sites of exchange involving morphotype Unknown 1 are presumed to be the palmetti–like ramifications of hyphae associated with a proportion of the host cortical cells. The nature of the nutritional benefits to the host of this frequent but rather casual relationship awaits investigation. It also remains to be seen how important this type of colonization by this fungus is under field conditions.

# Chapter 5 – A field bioassay to assess the impact of fire on ectomycorrhizal fungal communities in *Pinus halepensis* forests

## 5.1 Introduction

This chapter reports the results of an experiment in which seedlings of *Cistus creticus* were planted into burned and unburned forest areas in order to assess the impact of fire on the ectomycorrhizal communities of *Pinus halepensis* forests in central Greece.

Previous studies have shown that the post-fire ectomycorrhizal community can be explained by the colonizing strategies of the fungi involved. For example, ectomycorrhizal fungi colonizing *Pinus muricata* seedlings after a fire (*Rhizopogon, Wilcoxina* and *Tuber* species) were essentially the same as those colonizing seedlings grown in bioassays of the pre-fire forest soils suggesting that these species form fire-resistant spore banks (Baar *et al.*, 1999; Taylor & Bruns, 1999). These fungi were different to those found colonizing roots in soil cores taken before the fire which were dominated by *Tomentella, Russula* and *Lactarius* species (Taylor & Bruns, 1999). These fungi were also present after the fire but at reduced relative abundance (Grogan *et al.*, 2000; Horton *et al.*, 1998) suggesting that they were colonizing from slow-growing mycelial sources.

Grogan *et al.* (2000) suggested that the pattern of ectomycorrhizal species occurrence after fire appeared to be random with respect to space between seedlings. However the previous studies in the present thesis suggest that there is some spatial patterning in ectomycorrhizal inoculum that is related to resprouting shrubs (Chapter 3). It appears that most of the fungi colonizing naturally establishing *Cistus* seedlings after fires do so from vegetative mycelial sources that lose their colonizing potential when they are removed from the forest in soil cores (Chapter 4). Many of these are basidiomycetes that are apparently rare in the immediate aftermath of fire and tend to be located close to resprouting shrubs. It was suggested that these basidiomycetes are probably more abundant in the unburned forest but are reduced by the action of fire and in particular the consumption of forest litter where mycelia are concentrated. Thus, in the unburned forest where litter extends between trees and understorey shrubs, the spatial patterning of fungal inoculum observed in the burned forest is not expected to occur in the unburned forest.

While resprouting shrubs may be important as refugia for mycelia of these basidiomycetes after fires, it is still unclear exactly what the source of inoculum is. As it does not appear to

remain viable in soil removed from the site there would seem to be a requirement for an external resource base. The merits of resprouting ectomycorrhizal shrubs such as *Quercus coccifera* as providers of such a resource base have already been discussed (Chapter 3). However, there is also evidence to suggest that dead or dying roots can also maintain the colonizing potential of EM fungi (Ferrier & Alexander, 1985). Thus, as well as colonizing seedlings from newly colonized fresh root material produced by ectomycorrhizal resprouters, ectomycorrhizal fungi may also colonize from moribund roots of the mature pine trees killed by the fire. In order to separate these effects it was desirable to compare resprouter microsites with dead-pine microsites in burned areas.

To assess the impact of fire it is necessary to compare samples from burned and unburned areas within homogeneous forest stands. Ideally this would involve burning of forest plots in which the fungal community had been described before the fire. Studies of this kind are rare. Within Mediterranean-type climates, experimental burning is an extemely risky undertaking due to the extremely flammable nature of the vegetation and is very rarely considered. Therefore it was not possible to make a direct comparison of ectomycorrhizal fungi in the same forest plots before and after a fire. However forests in the Mediterranean are subject to frequent wildfires that are initiated by natural or anthropogenic events. Such fires can result in mosaics of burned and unburned forest stands within the landscape and these provide the basis for comparative studies.

Characterisation of ectomycorrhizal communities in undisturbed forest has typically involved sampling the roots of mature trees and shrubs by soil coring (Fransson *et al.*, 2001; Gardes & Bruns, 1996; Jonsson *et al.*, 1999a; Jonsson *et al.*, 1999b; Kernaghan, 2001; Taylor *et al.*, 2000; Taylor & Bruns, 1999b; Zhou & Hogetsu, 2002). This was not possible in the hard and rocky soils of the Mediterranean zone in central Greece. Hand excavation of roots is also extremely difficult in these soils and has the further disadvantage of being almost impossible to standardize among samples. Excavation of naturally establishing seedlings in burned and unburned forest stands would have been highly desirable but the target species, *Cistus creticus*, is a fire-following plant with hard-coated seeds that require the heat of wildfires to crack the coat and allow them to germinate. Though this may not to be an absolute requirement (Margarita Arianoutsou, pers. comm.), seedlings of this species very rarely establish naturally in unburned forests. Greenhouse bioassays of burned and unburned Mediterranean forest soils have previously been conducted with *Pinus halepensis* (Torres & Honrubia, 1997) and *Cistus creticus* seedlings (Chapter 4). However, greenhouse bioassays are biased towards fungi that readily colonize from spores and mycelial fragments. Particularly in undisturbed situations, many fungi can only colonize from mycelial sources

that are connected to active ectomycorrhizas (Fleming, 1984; Simard *et al.*, 1997). In such situations greenhouse bioassays may give misleading results if interpreted in isolation from field observations. Considering these factors, it was decided that a field bioassay conducted by planting pot-grown seedlings out into burned and unburned forest areas would be the most appropriate method of assessing the structure of the fungal communities present in the soil and thereby inferring the impact of fire.

Field bioassays more closely mimic natural conditions than greenhouse bioassays and have the added advantage of sampling the unburned forest with a host at the same developmental stage as that sampled in the burned forest. This may be important as one of the factors influencing inoculum potential of ectomycorrhizal fungi may be host carbohydrate supply.

Field bioassays also allow the investigator to manipulate the spatial distribution of the seedlings in order to test hypotheses. This field bioassay was designed to test the hypothesis that the EM fungal community structure in all unburned microsites is the same as that found around resprouting shrubs in burned areas but different to that found around dead pine trees and open patches in burned areas.

## 5.2 Methods

### 5.2.1 Seedling propagation

On 20 November 2000, *Cistus* seeds that had been collected from an area of unburned forest on the hill opposite the outplanting site were scarified with fine grade sandpaper, surface sterilised in 30% $H_2O_2$ for 15 minutes, rinsed in sterile $H_2O$ and then surface dried at 30 °C in an oven. Using sterile procedures, seeds were placed four to a pot (4 cm) on a 1:4 sieved peat:fine perlite mix that had been sterilised by autoclaving at 120 °C for one hour. The pots were then placed in a growth room under constant dark at 20 °C. Seedlings were removed to a greenhouse on emergence which occurred within three to four days. On 16 December 2000 the *Cistus* seedlings were thinned to one per pot.

From the time of first being removed to the greenhouse, all pots were watered once per week, alternating weekly between 20 ml of de-ionised water and a nutrient solution (50% Hoagland's No. 2 basal salt mixture, Sigma cell culture). The last application of nutrient solution was on 29 December 2000. Thereafter, each weekly watering was with de-ionised water only. Therefore all seedlings had been grown for six weeks without nutrient addition prior to outplanting. Seedlings were grown under natural light conditions and the maximum

and minimum temperatures in the greenhouse during the propagation period were 35 °C and 10 °C respectively.

### 5.2.2 Outplanting

#### *a) Pre-planting morphometric measurements made in greenhouse*

On 3 February 2001, from a starting stock of 200 seedlings, 64 were selected by eye for good health and uniformity of size. An additional three seedlings were selected to act as trial plants to be excavated towards the end of the allotted experimental period to assess whether there had been sufficient time for ectomycorrhizas to form. All selected seedlings were numbered and then randomly allocated to treatments.

In order to assess seedling uniformity at the start of the experiment, a number of measurements were made in the greenhouse prior to outplanting (see Figure 5.1).

1. Number of leaves. Leaves on primary and secondary shoots were recorded separately and are henceforth referred to as primary and secondary leaves.

2. Height

3. Basal diameter

4. Length of each of the 3rd pair of primary leaves (stem to leaf tip)

#### *b) Outplanting site*

The site selected for the outplanting experiment was located at the boundary of a large wildfire that occurred in August 2000 adjacent to the town of Malesina on the east coast of Central Greece approximately 120 km north of Athens (38° 35'N, 23° 15' E) (Figure 5.2). The site is a low-density natural stand of *Pinus halepensis* situated on a north-east facing slope of 25-30° gradient. The understorey is dominated by *Quercus coccifera*, *Pistacia lentiscus*, *Phyllirea latifolia*, *Olea europaea* and *Juniperus phoenicea*.

Table 5.1 shows the site characteristics that were recorded in the burned and unburned stands on 27 March 2001. Slope and tree height were measured with a (Carl Leiss altimeter). Tree height values are the mean of the eight trees selected as the plot marker trees in the burned and unburned areas (see below). Tree density was estimated from the number of trees occurring within a 10 m radius of the plot marker tree. All trees within the 10 m radius were measured for DBH.

Figure 5.1. Schematic diagram of a *Cistus creticus* seedling showing morphometric variables.

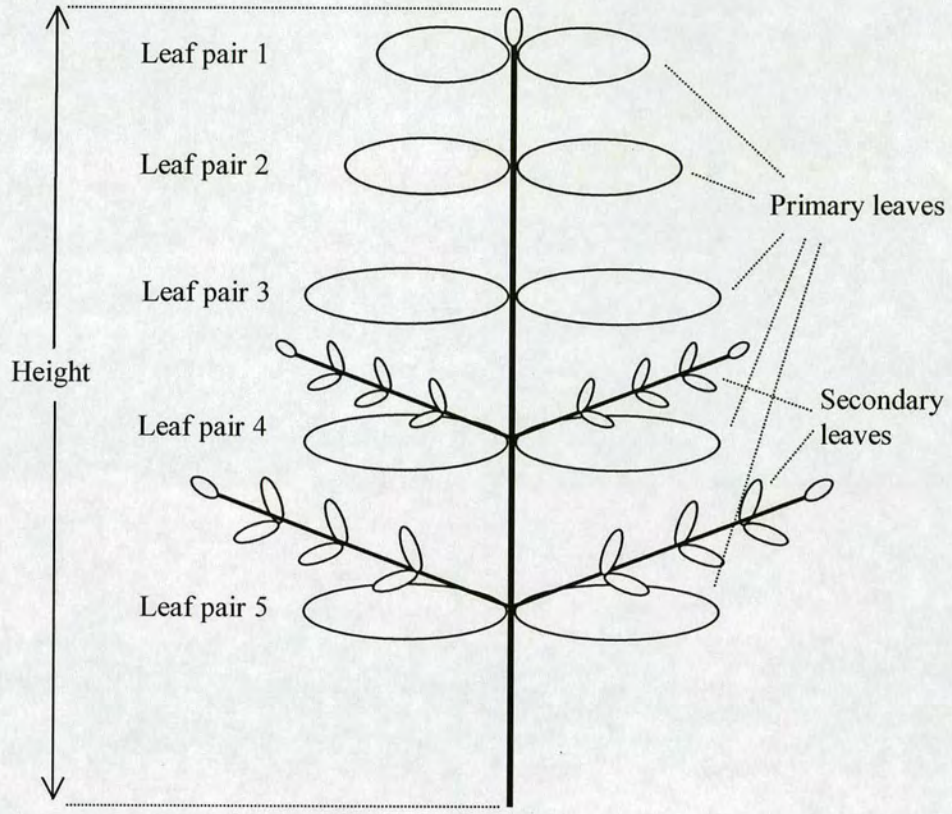


Figure 5.2. Map showing the location of the study area.

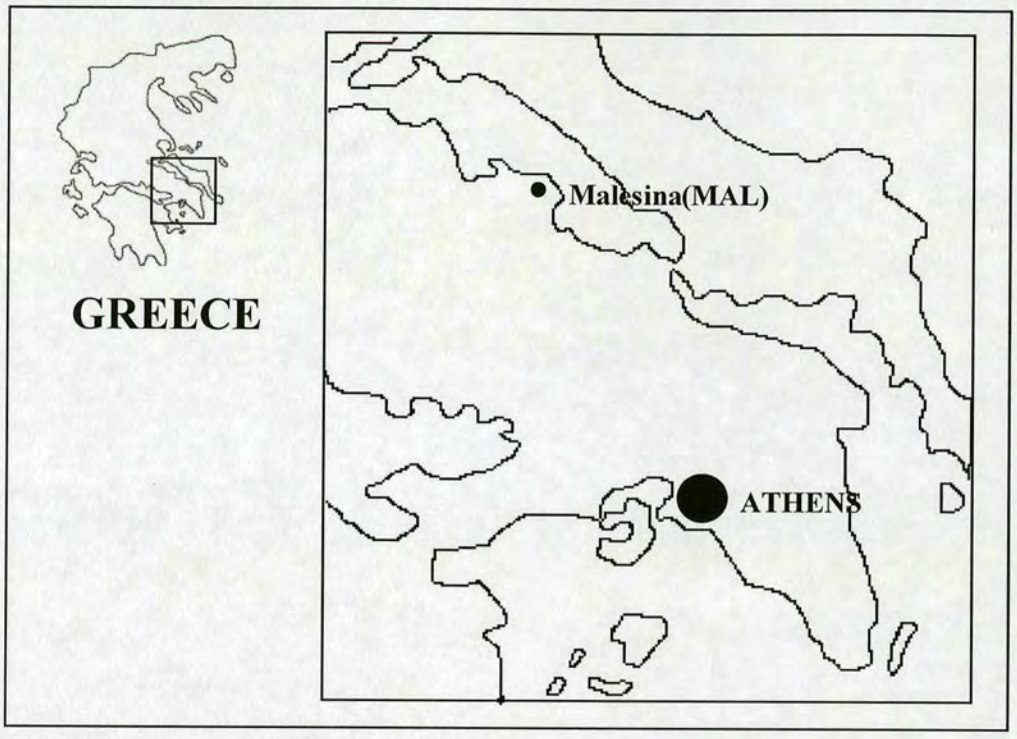



Table 5.1 Site characteristics measured at the outplanting site. Values are means (±s.e.) of the eight plots in each area (burned and unburned).

| | Slope (degrees) | Total No. trees | No. trees/ha (<9.5DBH) | No. trees/ha (>9.5DBH) | Mean DBH (trees >9.5) | Mean DBH (cm) (all) | Tree height (m) |
|---|---|---|---|---|---|---|---|
| Burned | 28 ±0.7 | 12.0 ±1.7 | 199 ±25 | 183 ±40 | 14.7 ±0.8 | 10.0 ±0.8 | 6.9 ±0.7 |
| Unburned | 27 ±1.2 | 15.4 ±4.7 | 298 ±107 | 191 ±47 | 18.6 ±2.2 | 12.3 ±1.8 | 7.5 ±0.6 |

### *c) Outplanting*

On 5 February 2001, eight plots were established in each of one unburned stand and one burned stand on either side of the fireline between the two. All of the pine trees in the burned plots were dead and all of the trees in the unburned plots were living. The plots were chosen by selecting mature pine trees of similar size. In each plot, in addition to the mature pine tree (henceforth referred to as "Pine"), one individual of each of an ectomycorrhizal shrub ["EM-

shrub"] (*Quercus coccifera*), a non-ectomycorrhizal shrub ["Non-EM-shrub"] (*Pistacia lentiscus*) and one open area ["Open"] were selected. "Pine", "EM-shrub", "Non-EM-shrub" and "Open" are henceforth, collectively referred to as 'microsites'. The criteria for selection were that the chosen microsites should be separated from each other by more than 3 m and that the open area should be at least one meter from the nearest resprouting stem.

Seedlings were randomly allocated to microsites in each of the burned or unburned plots and planted singly. The planting hole was made with a trowel and the seedlings were planted with a small plug of potting mix to moderate the shock of transplantation. Immediately after planting the seedlings were watered in with de-ionised water using a 7 litre horticultural sprayer at full pressure for 10 seconds (= approx. 80 ml). Immediately after watering, seedling height and basal diameter were re-measured.

To facilitate successful establishment and avoid mortality, on 10 February and 7 March 2001 all seedlings were watered with de-ionised water using the sprayer at full pressure for 5 seconds (= approx. 40 ml).

### 5.2.3 Monitoring seedling performance

#### *a) Seedling growth*

On 7 March and 29 April 2001, 30 and 83 days after outplanting respectively, *in situ* measurements were made of the morphological characters described in section 5.2.2a.

#### *b) Assessing variation in leaf light absorbance*

Loss of chlorophyll has long been used as an indicator of plant stress through nutrient deficiency and/or disease (Hendry *et al.*, 1987). Alteration of leaf optical properties associated with changes in chlorophyll concentration facilitates indirect, non-destructive estimation of leaf chlorophyll content and, thereby, degree of plant stress (Penuelas & Filella, 1998). Recent technological advances have enabled the easy measurement of leaf spectral properties and a clear linkage between plant stress, leaf chlorophyll content and leaf reflectance has been recently demonstrated (Carter & Knapp, 2001). Although leaf reflectance has been shown to be a better indicator of leaf chlorophyll content than leaf absorbance in some species (Richardson *et al.*, 2002), reflectance appears to be greatly affected by other leaf characteristics such as lamina thickness, water content and presence of trichomes (Baldini *et al.*, 1997). Therefore it was decided to measure leaf light absorbance as an indirect measure of variation in nutritional status in the outplanted *Cistus* seedlings.

Measurements were made with a Minolta SPAD-502 chlorophyll meter. This meter has a 0.06 $cm^2$ measurement area and calculates a chlorophyll index in SPAD units based on light absorbance at 650 and 940 nanometers. The claimed accuracy of the meter is ± 1.0 SPAD units (specifications reported in (Richardson *et al.*, 2002). For the purposes of the present study there was no requirement for estimation of absolute chlorophyll content and so no attempt was made to calibrate SPAD units against chlorophyll content for *Cistus creticus*. However, SPAD units recorded with this model of meter have been shown to correlate extremely well with total leaf chlorophyll in other species (Richardson *et al.*, 2002). Thus in the present study readings are recorded as SPAD units and it is assumed that relative variation in SPAD units reflects relative variation in leaf chlorophyll content.

Before recording leaf light absorbance in the field as a measure of plant performance it was necessary to assess the within-plant variation in this parameter and to ascertain the most appropriate part of the plant to measure. To do this measurements were made on plants in the greenhouse prior to outplanting.

On 30 January 2001, 25 of the pot-grown *Cistus* seedlings were randomly selected. Morphometric measurements were made as detailed in section 5.2.2a. To assess within-leaf variation measurements were made at three positions relative to the mid-rib on the leaf blade of one leaf of the 3$^{rd}$ primary pair (Figure 5.3a). All three positions were located 1 cm back from the leaf tip with Position 1 centred on the mid-rib and Positions 2 and 3 at distances of one and two millimetres from the mid-rib respectively. The 3$^{rd}$ primary leaf pair was selected for measurement as this was the uppermost of the fully expanded leaf pairs at any given time.

To assess within-plant variation light absorbance measurements were made on the 2$^{nd}$, 3$^{rd}$ and 4$^{th}$ leaf pairs. Measurements were made 1 cm back from the leaf tip and centered on the mid-rib (Position 1, Figure 5.3a).

Data were checked for homogeneity of variance and the residuals checked for normality. These criteria were met by both the leaf position data and the leaf pair data. Both sets of data were analysed by mixed model ANOVA with seedling entered as a random factor and leaf position or leaf pair as a fixed factor.

There was no significant difference between measurements made at Positions 2 and 3 within leaves but both of these had higher values than Position 1 ($F_{2,48} = 41.31$, $P < 0.001$) (Figure 5.3b). The top two leaf pairs (i.e, Leaf pair 2 and 3) did not differ significantly in SPAD

value. Both of these leaf pairs had significantly higher SPAD values than Leaf pair 4 ($F_{2,48}$ = 30.52, $P < 0.001$) (Figure 5.3c).

These results suggest that the measurement of either Position 2 or Position 3 on Leaf pair 2 or 3 provides a good estimate of maximum leaf chlorophyll content. It should be noted that this applies to seedlings grown in the greenhouse at optimal nutrient conditions.

#### *c) Measuring leaf light absorbance in the field*

Leaf light absorbance readings were taken from the outplanted *Cistus* seedlings on 7 March (30 days) and 5 April 2001 (59 days). Light absorbance was measured on all leaf pairs sufficiently large and healthy to accommodate the meter. In practice this meant leaf pairs 2, 3, 4, 5 and 6. The first pair was not sufficiently expanded and there was a tendency for the lower primary leaf pairs to have senesced. Light absorbance was measured at Leaf Position 2 (Figure 5.3a).

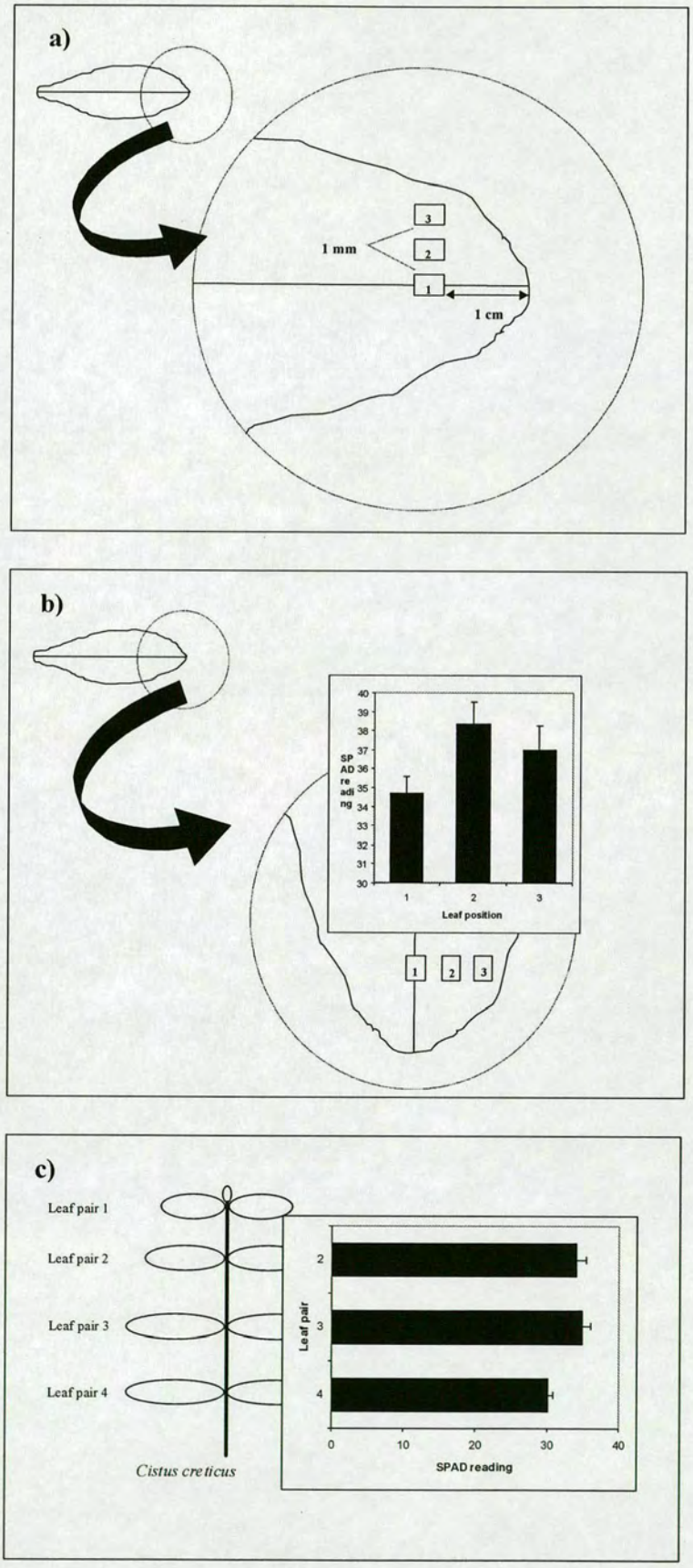
### 5.2.4 Harvest of seedlings and assessment of mycorrhizas

On 28-29 April 2001 (82-83 days), all outplanted seedlings were carefully excavated. Shoot fractions were separated from root fractions and placed in paper envelopes. The root fractions were wrapped in kitchen towel, soaked with water and placed in self-sealing plastic bags that were then placed in a cool box.

In the laboratory, root samples were gently washed under running tap water. Cleaned root samples were then stored at 4 °C in 2% glutaraldehyde until processed except for a 24 hour period during transport back to Scotland when samples were kept in cool boxes with ice packs.

For the assessment of mycorrhizal colonization, root systems were cut into fragments of approximately 1 cm length and randomly dispersed in water in a clear perspex tray measuring 17 x 11 cm, marked with a grid of 100 rectangles. All root tips occurring in 10 randomly selected grid squares were recorded as non-mycorrhizal or according to ectomycorrhizal morphotype.

Figure 5.3. Measurement of leaf light absorbance in pot-grown *Cistus creticus* seedlings. a) Measurement positions for assessing within-leaf variation. b) Light absorbance readings recorded at positions 1-3 on the leaf. c) Light absorbance readings recorded in leaf pairs 2, 3 and 4. Bars = 95% confidence intervals.



Due to the difficulty of distinguishing moribund and living root tips in many cases, all root tips were counted to avoid the imposition of subjective judgements upon the data. The remainder of each root system was scanned under the microscope and the presence of any additional morphotypes was recorded. The number of morphotypes counted in the 10 grid squares is referred to as 'number of morphotypes (sub-samples)' and those counted in the whole root system as 'number of morphotypes (total). For each morphotype, percentage colonization (i.e., percentage of total root tips colonized), relative abundance (i.e., percentage of colonized root tips) and frequency (number of seedlings were calculated from the sub-sample counts.

### 5.2.5 Statistical analysis

The ectomycorrhizal morphotype data were ordinated by non-metric multi-dimensional scaling (NMDS) using PC-ORD for Windows (McCune & Mefford, 1997). The raw data were relative abundance (i.e., percentage of colonized root tips) of all morphotypes occurring in three or more samples (= 11 morphotypes). Prior to ordination data were arcsine square-root transformed. Within the NMDS ordination routine in PC-ORD (labelled NMS), the Sørensen distance measure was selected. Sørensen distance in PC-ORD is measured as percent dissimilarity (PD) (McCune & Mefford, 1997). The distance between objects j and k is:

$$PD = 100 (1 - [\Sigma |A_{ij} - A_{ik}| / (\Sigma A_{ij} + A_{ik})])$$

Where $A_{ij}$, $A_{ik}$ = number of individuals (root tips) of morphotype i in each sample j and k, n = number of morphotypes in each sample. As expressed in PC-ORD, Sørensen distance (PD) is the same as Bray-Curtis similarity expressed as a percentage. Sørensen is more usually associated with an index of similarity based on presence or absence data.

The starting co-ordinates were random numbers and the maximum number of iterations was 100 with an initial step length of 0.20. The data were ordinated with six axes initially. Inspection of the scree plot of axis against stress showed that there was little further reduction in stress beyond three axes. The data were re-ordinated with three axes and displayed as a two-dimensional plot of the axis combination that showed the clearest separation of samples.

To test the null hypothesis that there was no influence of fire or microsite on ectomycorrhizal community structure, a permutation test based on the ranked similarities between samples was carried out using the ANOSIM routine in PRIMER v.4.0 (Plymouth Marine Laboratory)

(Clarke, 1993). The test was carried out on the ranked similarity matrix generated by Bray-Curtis distance measures for arcsine square-root transformed relative abundance data. Fire and microsite were treated as two factors in a two-way crossed design with two ('burned', 'unburned') and four ('Open','Pine', 'Non-EM shrub', 'EM shrub') levels respectively. The sample statistic (R) is calculated as

$$R = (r_b - r_w) / (M/2)$$

where $r_b$ and $r_w$ = the average of the rank similarities of all pairs of samples between and within treatments respectively, M = n(n-1)/2 where n = total number of samples (Clarke, 1993). R for the observed data was then compared against the distribution of R calculated from a maximum of 5000 permutations randomly selected from the total number of possible permutations for each factor.

The effects of fire and microsite on percentage of root tips colonized, number of morphotypes per seedling (sub-samples and total) and increment of seedling growth parameters (primary leaves, secondary leaves, height, basal diameter) were analysed using two-way, mixed model, nested ANOVA with fire and microsite as fixed factors. Plot was considered as a random factor nested within burned and unburned stands. Percentage data were arcsine square-root transformed prior to analysis. Data were checked for homogeneity of variance (Bartlett's test) and residuals checked for normality (Anderson-Darling test). For the growth parameters measurements made at the start of the experimental period were entered as covariates. Due to seedling mortality and herbivory of some shoots the design was unbalanced to varying degrees for the parameters tested and the data were therefore analysed using the Generalised Linear Model routine in MINITAB. The effects of both fire and microsite were tested against the nested plot-level error due to the unbalanced design. The structure of the ANOVA and degrees of freedom for each parameter are given in Table 5.2, columns A and B).

The mean light absorbance for each leaf pair in the burned and unburned plots was graphed with 95% confidence intervals (see Figure 5.10). Inspection of the graph suggested that leaf pairs 2, 3 and 4 varied between burned and unburned plots while leaf pairs 5 and 6 did not. To simplify the analysis, the values for leaf pairs 2, 3 and 4 were pooled by taking the arithmetic mean. The effects of fire and microsite on the pooled leaf light absorbance were then analysed as for the other parameters (see Table 5.2, column C for ANOVA structure and degrees of freedom). As the interaction between fire and microsite in the overall analysis of pooled leaf light absorbance was weak and significant ($F_{3,14} = 1.73$, $P = 0.207$), the effect

of microsite was analysed within the burned and unburned plots separately with plot entered as a random factor (Table 5.2, columns D and E).

Table 5.2 ANOVA structure and degrees of freedom (df) for parameters analysed. A – percentage of root tips colonized, number of morphotypes per seedling (sub-samples and total). B – growth increment parameters (number of primary and secondary leaves, height and basal diameter). C – Leaf light absorbance (mean of leaf pairs 2, 3 and 4). D – Leaf light absorbance (mean of leaf pairs 2, 3 and 4) in burned plots. E - Leaf light absorbance (mean of leaf pairs 2, 3 and 4) in unburned plots.

| Source | df | | | Source | df | |
|---|---|---|---|---|---|---|
| | A | B | C | | D | E |
| Covariate | - | 1 | - | Microsite | 3 | 3 |
| Fire | 1 | 1 | 1 | Plot | 7 | 7 |
| Microsite | 3 | 3 | 3 | Error | 16 | 17 |
| Fire x Microsite | 3 | 3 | 3 | Total | 26 | 27 |
| Plot(Fire) | 14 | 14 | 14 | | | |
| Error | 35 | 28 | 33 | | | |
| Total | 56 | 50 | 54 | | | |

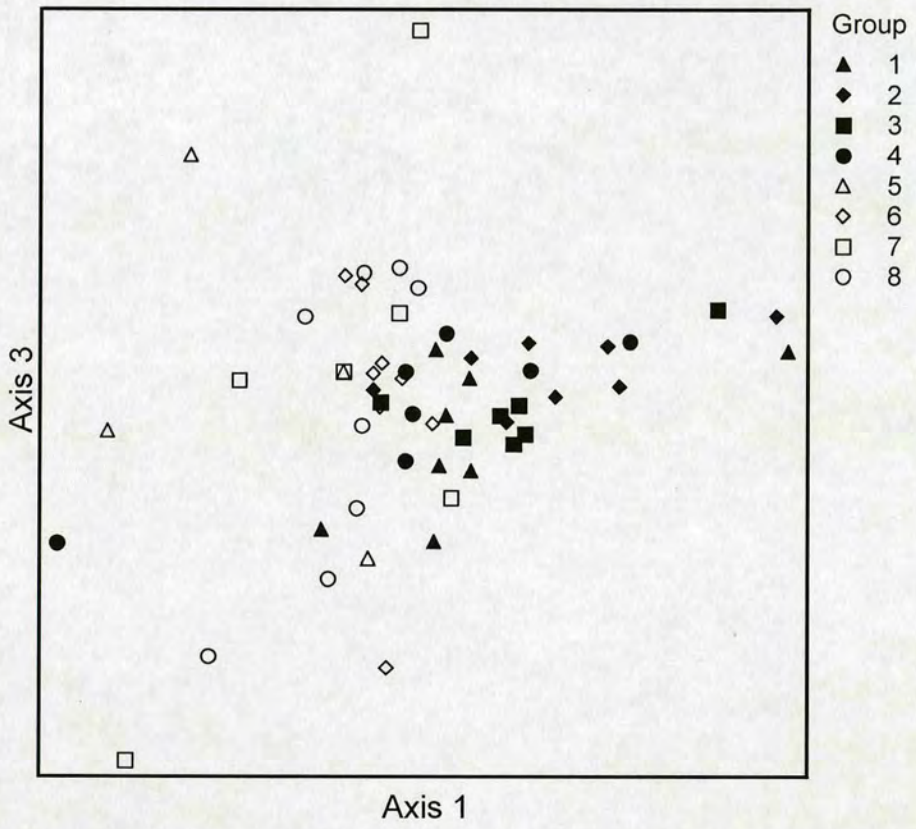
## 5.3 Results

### 5.3.1 Ectomycorrhizal fungal community structure

A total of 17 morphotypes was recorded on the outplanted *Cistus* seedlings. Two of these ('Unknown 5' and 'Unknown 2') were only recorded as present in the survey of the remainder of roots after the sub-samples had been assessed. A further four morphotypes ('Ascomycete 1', '*Tuber* 3', '*Tuber* 1', 'Unknown 12') occurred on less than three seedlings. These six morphotypes together were excluded from the NMDS ordination and ANOSIM analyses.

The NMDS ordination plot in Figure 5.4 shows a separation of seedlings from burned and unburned plots with respect to ectomycorrhizal fungal community structure. Burned samples tend to be closer to one another in ordination space than unburned samples suggesting that there was less variation in ectomycorrhizal community structure between seedlings planted into burned forest compared to those planted into unburned forest. However, the stress values associated with the ordination are relatively high (3-D stress = 0.16, 2-D stress = 0.24) and should be treated with some caution.

Figure 5.4 Plot of NMDS ordination of ectomycorrhizal fungal communities associated with *Cistus creticus* seedlings planted at four microsites in burned and unburned forest stands in central Greece. Data are relative abundance for morphotypes occurring in more than 2 samples. Ordination was carried out on three axes (3-D stress = 0.16). Plot is 2-dimensional representation of sample similarity on axes 1 and 3 (2-D stress = 0.24). (Δ) 'Open'; (◊) 'Pine'; (□) 'Non-EM shrub'; (O) 'EM shrub'; filled symbols indicate burned samples.




Two-way ANOSIM indicated a significant effect of fire on community structure (R = 0.429, $P < 0.01$) but no overall effect of microsite (R = 0.032, $P = 0.20$). One-way ANOSIM indicated that there was a significant difference between microsites within the burned plots (R = 0.094, $P = 0.04$) but not the unburned plots (R = -0.043, $P = 0.73$). Pairwise tests between microsites showed that differences between Pine and EM-shrub (R = 0.394, $P = 0.006$) and between Non-EM-shrub and EM-shrub (R = 0.196, $P = 0.04$) were significant while other comparisons were not.

Based on the sub-sample counts, the ectomycorrhizal communities of *Cistus* seedlings planted into both burned and unburned plots were dominated by morphotypes 'E-Strain' and 'Unknown 4' (Figure 5.5). 'E-Strain' was very widely distributed, occurring at all microsites (Figure 5.6) and competing well with other morphotypes (39.0% ± 4.1 s.e. relative abundance). 'Unknown 4' was less frequent, particularly in the burned plots where it was disproportionately reduced in frequency at Pine and Non-EM-shrub compared to Open and EM-shrub microsites (Figure 5.6). Where present, Unknown 4 also competed well (45.7% ± 5.2 s.e. relative abundance).

'Unknown 1' was also very common in the burned plots where it was present equally at all microsites (Figure 5.6) but was not recorded at all from the unburned plots (Figure 5.5). '*Tuber* 3' was the only other morphotype found exclusively in the burned plots. While it was very infrequent, occurring on only two seedlings, where present it was capable of competing well with other morphotypes (36.3% relative abundance). 'Ascomycete 2', 'Unknown 14', '*Inocybe* 2' and 'Unknown 12' were found exclusively in the unburned plots. Of these 'Ascomycete 2' was the most frequent and the most competitive (41.4% ±12.8 s.e. relative abundance).

The frequency of confirmed basidiomycetes *Thelephora terrestris*, Thelephoroid 2, Basidiomycete 5 and *Inocybe* 2 was reduced in the burned plots (Figure 5.5). The only other confirmed basidiomycete, Basidiomycete 4, occurred at slightly higher frequency in the burned plots compared to the others but its relative abundance was much reduced compared to the unburned plots. While *T. terrestris* and Basidiomycete 4 were recorded only at shrub microsites in the burned plots, Thelephoroid 2 and Basidiomycete 5 were recorded at shrub and open microsites (Figure 5.6).

The K-dominance plot presented in Figure 5.7 shows that burning resulted in an increase in dominance within the associated ectomycorrhizal fungal communities. The cumulative percentage of colonised root tips (i.e., relative abundance) attributed to the three most abundant morphotypes shifted from 73.7% in unburned forest ('Unknown 4', 'Ascomycete 2', 'E-Strain') to 92.0% in burned forest ('Unknown 1', 'E-Strain', 'Unknown 4').

Figure 5.5 Effect of burning on frequency (solid bars), relative abundance (open bars) and percentage colonization (shaded bars) of morphotypes colonizing outplanted *Cistus creticus* seedlings. Error bars = standard error where n > 2.

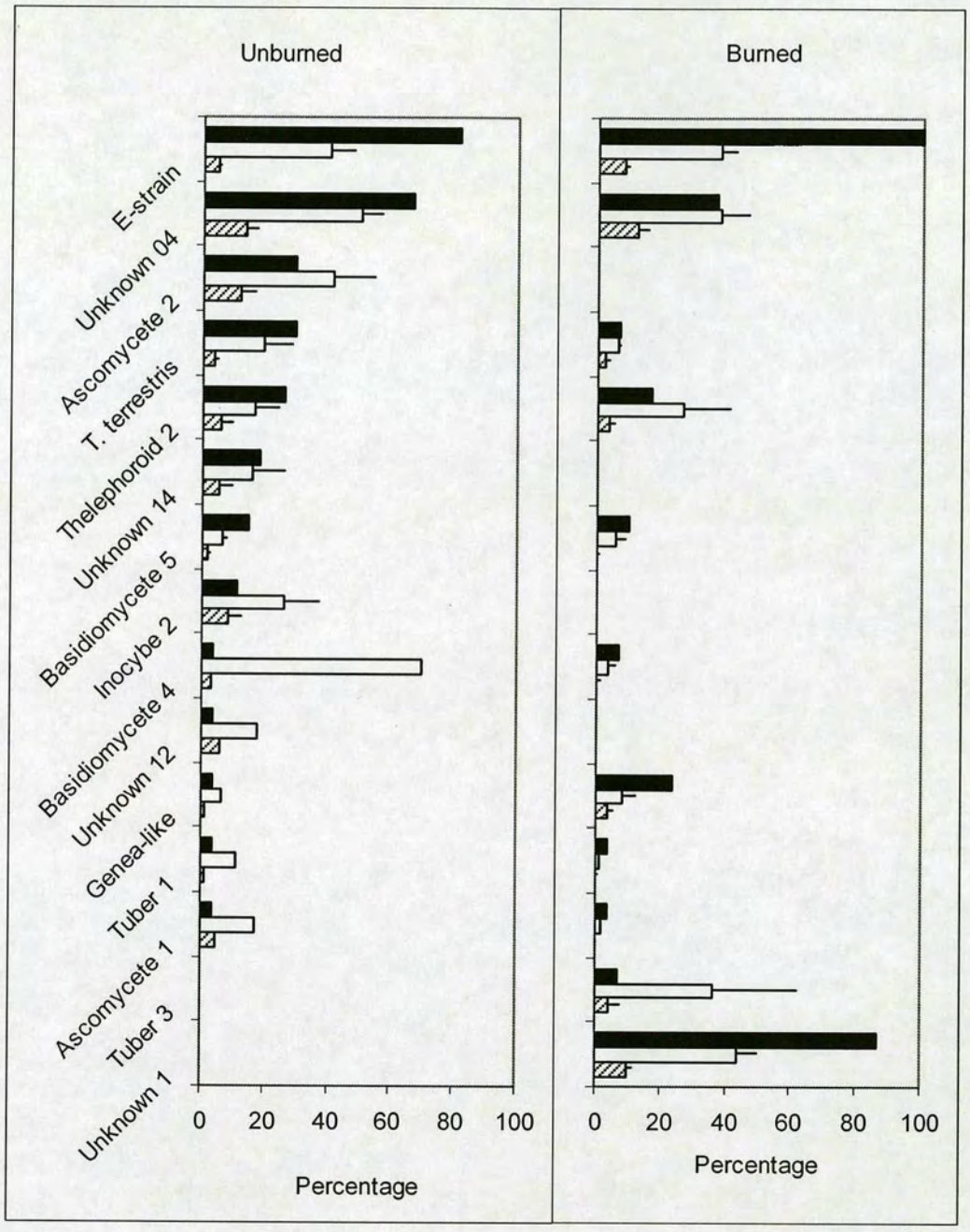


Figure 5.6 Effect of microsite on frequency of occurrence of morphotypes colonizing outplanted *Cistus creticus* seedlings (Black bars = EM-shrub, cross-hatched bars = Non-EM-shrub, shaded bars = Pine, open bars = Open). Frequency is expressed as the percentage of seedlings in which a morphotype was present.

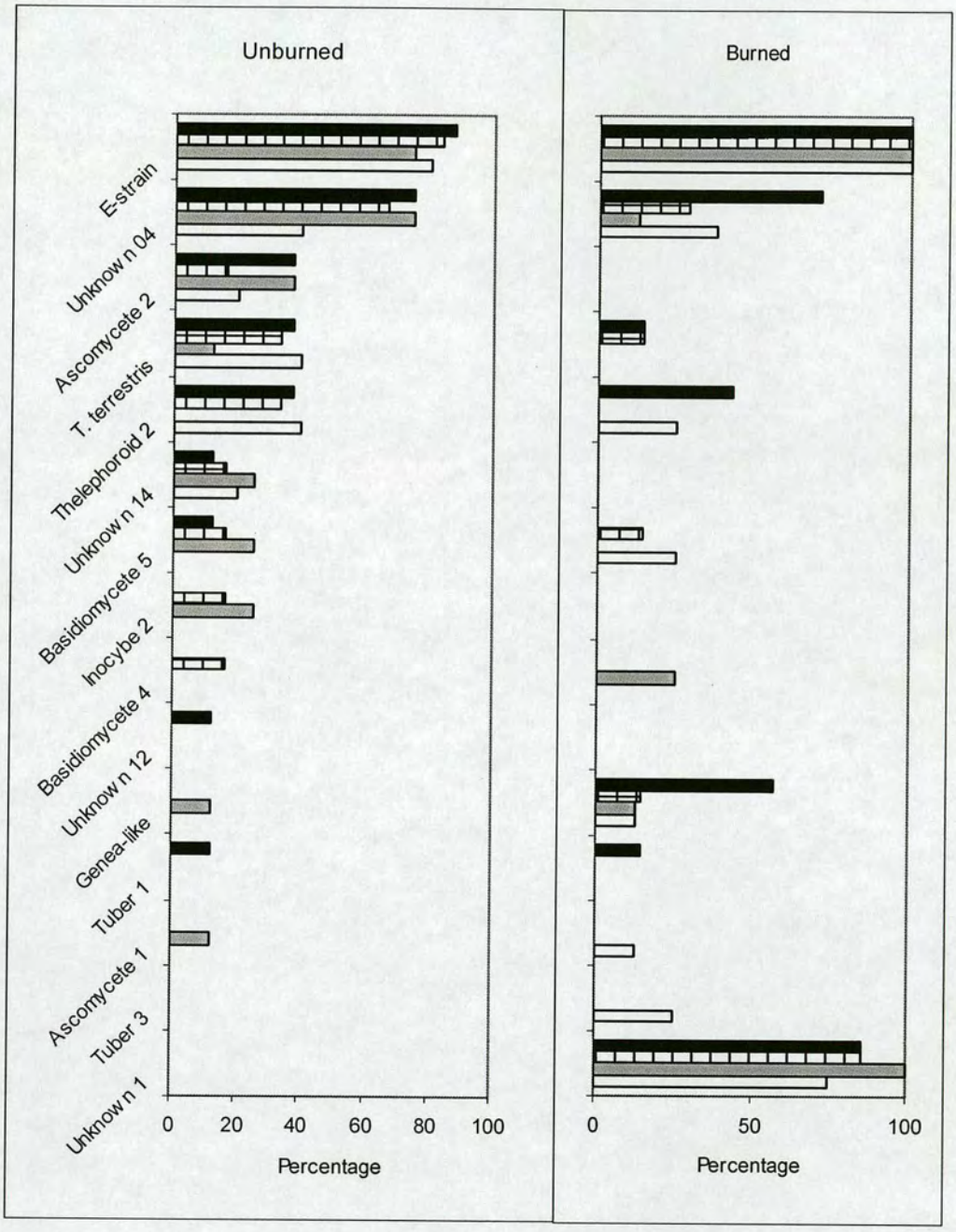
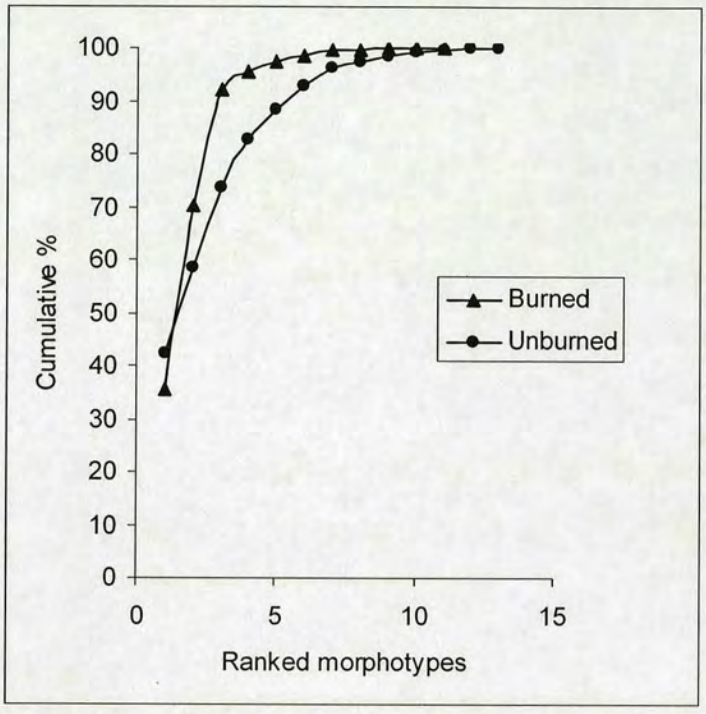


Figure 5.7 K-dominance plots for number of root tips colonized by ectomycorrhizal fungi in *Cistus creticus* seedlings planted in burned and unburned forest areas in central Greece.




### 5.3.3 Mycorrhizal colonization

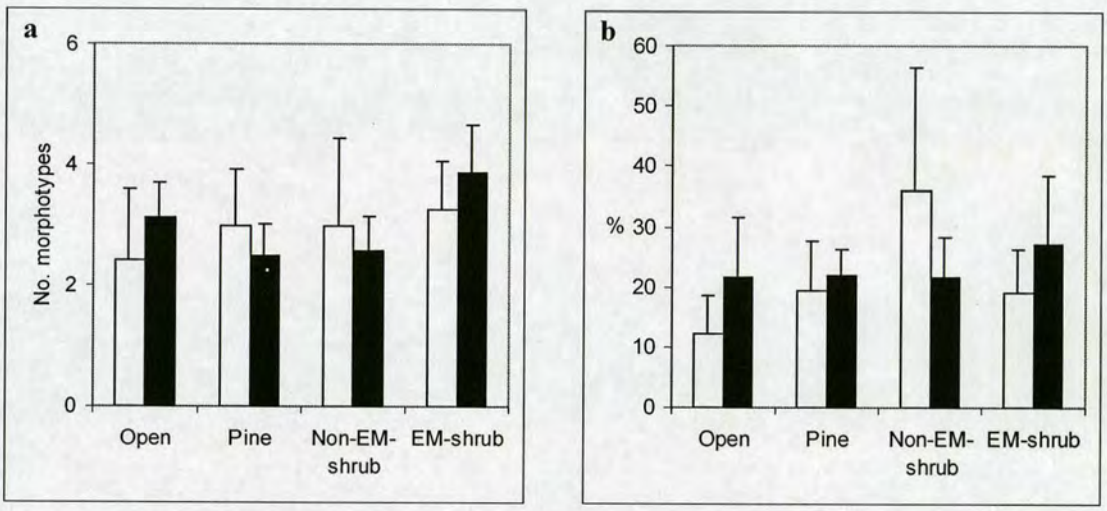
Fire and microsite had no significant effect on number of morphotypes per seedling or percentage of root tips colonized (Figure 5.8a, b) of outplanted *Cistus creticus* seedlings. Though insignificant, the trend was towards greater numbers of morphotypes per seedling at the Open and EM-shrub microsites in the burned plots while at the Pine and Non-EM-shrub microsites there were greater numbers in the unburned plots (Figure 5.8a). There was a trend towards greater percentage of root tips colonized in burned compared to unburned plots at all microsites except Non-EM-shrub. Percentage colonization was particularly variable at Non-EM-shrub microsites in unburned plots.

### 5.3.4 Seedling growth

*Cistus creticus* seedlings planted into burned plots produced larger numbers of secondary leaves ($F_{1,14} = 16.81$, $P < 0.001$) and showed greater expansion of the stem basal diameter ($F_{1,14} = 24.86$, $P < 0.001$) during the outplanting period than seedlings planted into unburned plots (Figure 5.9b, d). Though not statistically significant, the trend was for seedlings to

grow taller in the unburned plots compared to burned plots (Figure 5.9c). Microsite had no effect on any of the growth parameters which also did not co-vary significantly with their start values. Though again statistically insignificant, there was an interesting trend towards greater growth at the Open microsites, particularly in terms of secondary leaf production and stem basal diameter in the burned plots (Figure 5.9).

Figure 5.8 Effect of fire and microsite on a) number of morphotypes per seedling (total), b) percentage of root tips colonized by ectomycorrhizal fungi in *Cistus creticus* seedlings planted in burned and unburned forest plots in central Greece. Values are means. White bars = unburned, black bars = burned. Error bars = 95% confidence intervals.



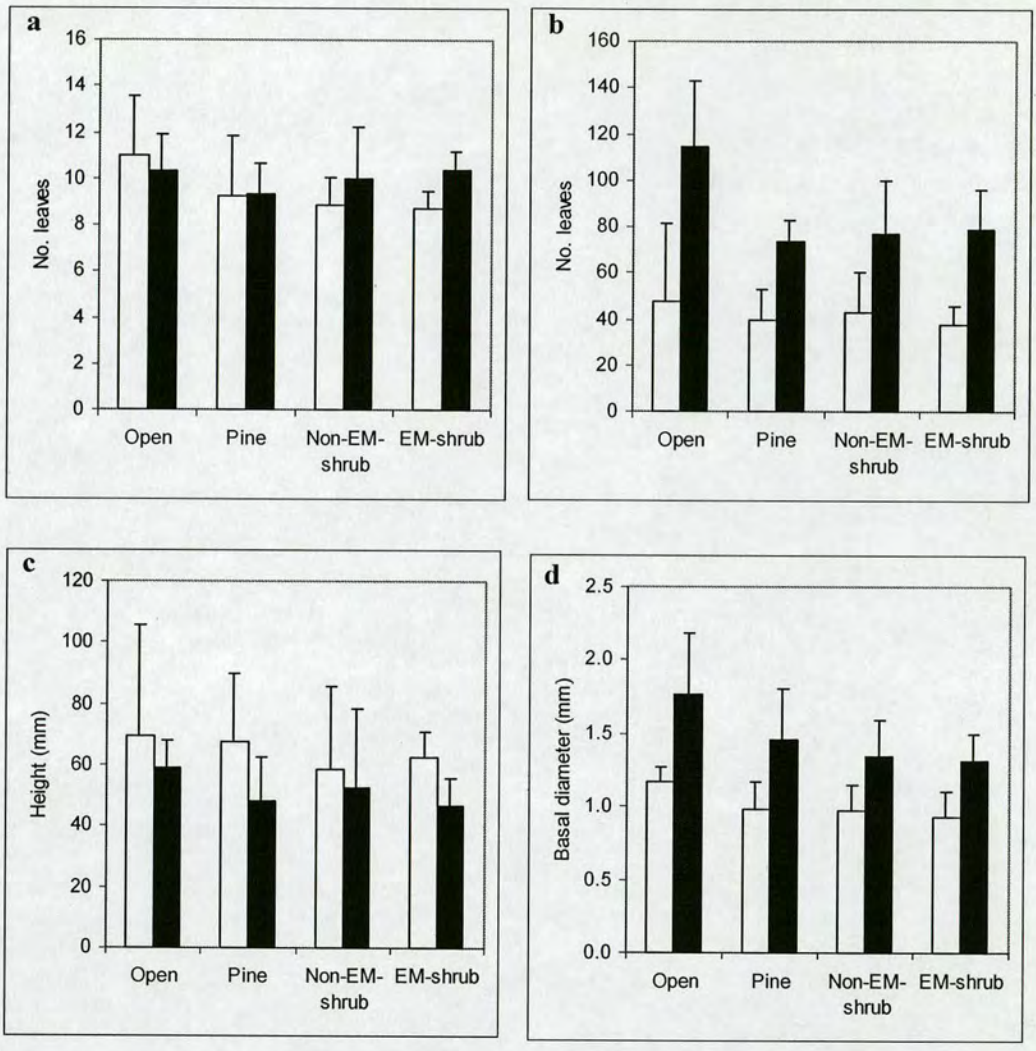

### 5.3.5 Leaf light absorbance

Figure 5.10 clearly shows that in the burned plots, the upper three leaf pairs of *Cistus creticus* seedlings had higher levels of light absorbance compared to those of seedlings planted into unburned plots but that leaf pairs 5 and 6 were not significantly affected. Analysis showed that light absorbance pooled across leaf pairs 2, 3 and 4 was significantly higher in the burned plots compared to the unburned plots ($F_{1,14} = 34.25$, $P < 0.001$). The interaction between fire and microsite for pooled light absorbance was small and insignificant ($F_{3,14} = 1.73$, $P = 0.207$) so the effect of microsite was analysed within burned and unburned plots separately.

Microsite had no significant effect on leaf light absorbance within burned plots or unburned plots. Within burned plots seedlings planted next to dead pine trees trended towards higher

leaf light absorbance values compared to those planted in open areas and next to non-EM resprouters (Figure 5.11).

Figure 5.9 Effect of fire and microsite on change in a) number of primary leaves, b) number of secondary leaves, c) stem height and d) stem basal diameter for *Cistus creticus* seedlings planted out in burned and unburned forest plots in central Greece. Values are means. White bars = unburned, black bars = burned. Error bars = 95% confidence intervals.




## 5.4 Discussion

Burning resulted in a change in the structure of the ectomycorrhizal fungal community through a shift in species composition and an increase in species dominance in the burned forest.

Figure 5.10 Effect of burning on light absorbance of successive leaf pairs of *Cistus creticus* seedlings planted in burned and unburned forest areas in central Greece. Readings taken 8 weeks after outplanting. Leaf pairs run from top (pair 2) to bottom (pair 6). Values are means, bars = 95% confidence intervals.

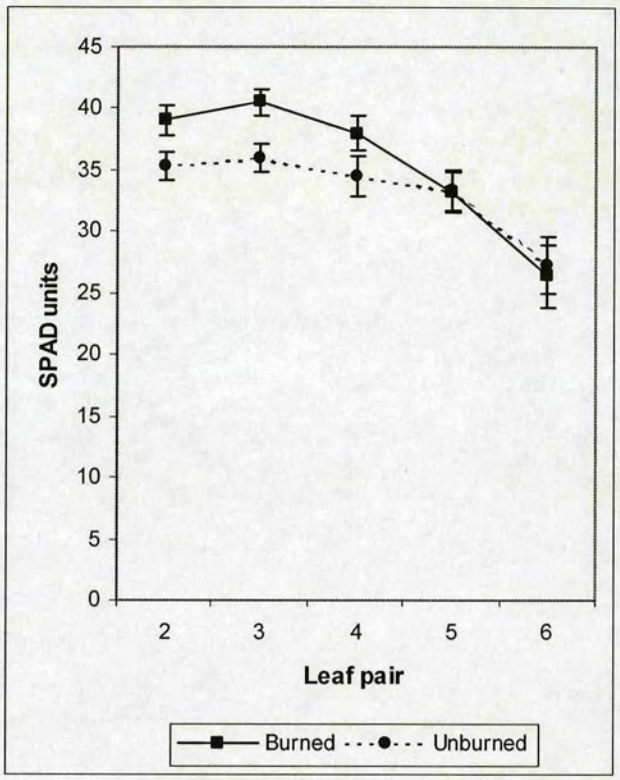
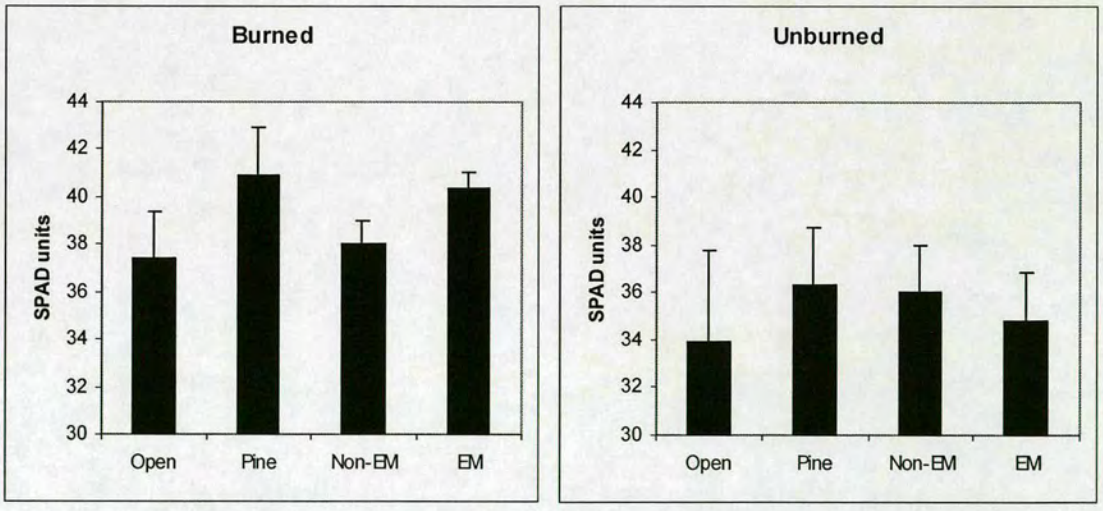



Figure 5.11 Effect of microsite on leaf light absorbance within a) burned and b) unburned forest plots. Data are arithmetic mean of leaf pairs 2, 3 and 4. Error bars = 95% confidence intervals.



The most notable change in species composition was the appearance of the morphotype 'Unknown 1' at high frequency and relative abundance in the burned plots compared to its complete absence from the unburned plots. This was somewhat unexpected as this same morphotype was also found extensively on seedlings grown in both burned and unburned forest soils in a greenhouse bioassay (Chapter 4). This indicated that inoculum of this fungus is present in forest soils prior to burning and that it has no requirement for burning to render the inoculum infective. Unknown 1 was also common in the upper few centimetres of the root systems of *Cistus* seedlings establishing naturally in burned forest stands (Chapter 3). It is notable that both there and in the greenhouse bioassay there were few other morphotypes co-occurring. It may be that Unknown 1 was excluded from colonizing the outplanted seedlings in the unburned plots in the present study by the presence of other fungi in the upper soil layers. In the burned plots these fungi are eliminated or reduced in the upper soil layers allowing Unknown 1 to colonize the seedlings there, assuming that Unknown 1 inoculum is heat resistant to some degree.

In contrast to the case of 'Unknown 1', the E-strain morphotype was dominant in both unburned and burned plots, colonizing virtually all of the seedlings in the study. Like Unknown 1, E-Strain was also common in the upper parts of root systems in naturally establishing seedlings but this fungus, or group of fungi, evidently is not constrained in unburned forest soils.

Confirmed basidiomycete fungi were generally reduced in frequency after the fire but none were very common in the unburned forest in the first place. This was unexpected. Many if not most of the basidiomycetes in the unburned forest may reasonably be assumed to exhibit ecological characteristics of late-stage fungi, i.e., colonize predominantly from mycelia attached to established mycorrhizas. Their low occurrence reported here might be the result of disturbance to mycelial networks caused by the process of planting the seedlings. With the colonizing potential of late-stage fungi thus reduced, fungi readily colonizing from isolated sources present in the soil would have an advantage. This would explain the high abundance of the E-strain morphotype on the seedlings planted into the unburned forest plots. The most frequent of the basidiomycetes in the unburned forest plots was *T. terrestris* and this colonized approximately 30% of the seedlings planted there. *Thelephora terrestris* is considered to be an early-stage fungus that can colonize readily from spores which may explain why it was more abundant in the unburned forest than other basidiomycetes. Both *T. terrestris* and *Inocybe* 2 (another early-stage genus) were severely reduced in frequency by burning, to zero in the latter case. The other basidiomycetes maintained the same low frequency in burned plots as in unburned plots. It might be the case that early-stage fungi are

low in abundance on the roots of mature plants in undisturbed forest where they have to compete with late-stage fungi. However, by maintaining large spore banks in the soils of the unburned forest they can take advantage of situations where the soil becomes disturbed. As these are epigeous fungi, spores will be concentrated at the soil surface and therefore vulnerable to destruction by fire. Therefore, after fires, spore inoculum is reduced and mycelial inoculum is low and thus their overall frequency among newly establishing seedlings is reduced. E-strain fungi probably behave in much the same way as other early-stage fungi in undisturbed forest but may have the advantage of fire-resistant spores or sclerotia which would explain their abundance in the burned plots.

The possibility of disturbance to fungal mycelia by the act of planting seedlings is one of the disadvantages of the field bioassay which lies somewhere between the greenhouse bioassay and observation of naturally establishing seedlings in its mimicry of the natural system.

Species richness trended towards being higher at Open and EM-shrub microsites and lower at Pine and Non-EM-shrub microsites in the burned plots compared to the unburned plots. Interestingly, the frequency of occurrence of Unknown 4 which was common at all microsites in unburned plots appeared to be disproportionately reduced at Pine and Non-EM-shrub microsites in burned plots. Reduced species richness and abundance of some fungi at Pine and Non-EM-shrub microsites may be related to an adverse effect of ash quantity and/or quality either indirectly via an effect on seedling growth or directly via soil chemical changes that affect the fungi themselves. *Cistus* seedling growth can be negatively affected by large amounts of ash, possibly through increased osmotic stress or toxic levels of some ions (Ne'eman *et al.*, 1993). This would explain why, in the burned plots in the present study, seedlings planted into open areas where ash is sparse appeared to produce more secondary leaves and a greater increment in stem basal diameter than seedlings establishing at other burned microsites though the high level of variation meant this was not statistically significant. However, species richness and Unknown 4 abundance were also greater at the EM-shrub microsites where ash quantities were as high (personal observation) and seedlings as big as at pine and non-EM shrub microsites and in any case seedling size does not seem to affect associated mycorrhizal diversity (Chapter 3). It may be that ash quality under pine and *Pistacia lentiscus* (Non-EM-shrub) was in some way detrimental to some fungi. (Kutiel & Shaviv, 1992) demonstrated that forest soils burnt experimentally with *Pinus halepensis* leaves and twigs contained more ammonium and nitrate than soils burnt with *Quercus calliprinos* leaves and twigs. As nitrogen ions are a requirement for chlorophyll synthesis, this is consistent with the finding in the present study that seedlings growing under burned pine trees had the highest levels of leaf light absorbance of all the microsites though again

this was not statistically significant. It also indicates that seedling growth is probably not limited by availability of nitrogen ions as seedlings in open areas grew more but had lower leaf light absorbance levels compared to seedlings under the burned pine trees.

These findings suggest variation in ash quality between microsites but further studies will be required to define differences in more detail and to examine the effects of such variation on mycorrhizal fungi. Nevertheless, this highlights the possibility that structuring in ectomycorrhizal fungal communities after fires may not be entirely explained by differences in colonizing strategies and sources of inoculum. The interplay between EM colonization and heterogeneity in ash quantity and quality is likely to be complex. Further work in which EM colonization is linked with assessments of soil nutrients in burned forests is required to begin teasing apart these complex relationships.

# Chapter 6 – Temporal variation in early post-fire colonisation of naturally establishing *Cistus creticus* L. seedlings by ectomycorrhizal fungi.

## 6.1 Introduction

This chapter reports the results of a series of excavations carried out to trace the development of the EM community associated with naturally establishing *Cistus creticus* seedlings during their first five months of growth after a wildfire.

Previous studies on post-fire EM community structure have tended to commence at least one year after the fires (Baar *et al.*, 1999; Danielson, 1984b; Grogan *et al.*, 2000a; Jonsson *et al.*, 1999a; Mah *et al.*, 2001; Miller *et al.*, 1998; Stendell *et al.*, 1999; Visser, 1995). By this time changes in the fungal community may have already occurred making it unclear what the initial stages of colonization were like. In one of the few studies that has looked at early post-fire colonization, *Pinus muricata* first formed EM root tips in the third month after germination (Horton *et al.*, 1998). The same pattern has also been reported for *Pinus contorta* seedlings establishing after fires in Wyoming, USA (Miller *et al.* 1998).

The delay in EM formation until the third month in pine seedlings is likely to be a function of seedling development. Fuelled by reserves from their relatively large seeds, *Pinus halepensis* seedlings tend to direct their initial growth towards the development of a taproot (personal observation). The relatively large amounts of seed-stored nutrients allow pines to remain independent of a need to acquire external sources of minerals for relatively long periods (Hanley & Fenner 2001). *Cistus creticus* on the other hand, has a much smaller seed than pines and is therefore nutritionally challenged much earlier in its development (Hanley & Fenner, 1997). Thus *Cistus* seedlings may be driven towards symbiotic partnership with EM fungi earlier in their development.

Previous observations of naturally establishing *Cistus* seedlings four months after germination showed that they were dominated by the E-Strain and Unknown 1 morphotypes occupying the upper few centimetres of root systems (Chapter 3). It was hypothesised that, given their position on root systems, these morphotypes represented the earliest EM formations after seed germination. However this need not necessarily have been the case and therefore it was necessary to assess the presence of these and other fungi in the first months after seed germination.

Unknown 1 exhibited an unusual pattern of colonization in greenhouse bioassays of forest soils (Chapter 4). As well as colonizing root tips, this fungus was found extensively in the long roots where it formed patchy mantle-like and Hartig-net-like structures. It was noted that artificial conditions associated with the greenhouse environment may have resulted in unrepresentative behaviour of this fungus. It was therefore desirable to investigate whether this pattern of colonization also occurs in nature and if so at what stage after host seed germination.

The previous assessment of naturally establishing seedlings (Chapter 3) showed that percentage colonization varied greatly between *Cistus* seedlings. For example, the percentage of root tips colonized in seedlings bearing a single morphotype ranged from 6.7% to 100% (Figure 3.4). This did not appear to be related to seedling size. It seems that other factors determine the level to which individual fungi colonize individual seedlings. The results of numerous experiments carried out under controlled laboratory conditions suggest that mycorrhizas are most readily formed under conditions of low soil nutrients (Smith & Read, 1997). It is also known that EM fungi respond differently to source and concentration of major nutrients under laboratory conditions (Dickie *et al.*, 1998). Thus where major nutrients vary in nature, it is expected that fungal responses and mycorrhizal colonization may vary also. It is clear that ash deposition after fires is heterogeneous and influenced by the structure and composition of the forest vegetation and this in turn results in a patchy nutrient environment (Kutiel & Shaviv, 1992; Ne'eman *et al.*, 1992). Such conditions provide an ideal system within which to investigate relationships between seedling growth, EM fungal colonization and soil nutrients at the plant level.

Thus this study was conceived with the aim of investigating the following hypotheses. 1) *Cistus* seedlings establishing in post-fire forest soils form fully developed EM associations early in their establishment phase. 2) The EM community changes with time and total colonization levels and colonization by individual morphotypes can be related to levels of soil nutrients. 3) The patterns of diffusive mycorrhiza-like colonization of long roots observed in a greenhouse bioassay are also common in naturally establishing seedlings.

## 6.2 Methods

### 6.2.1 Study site

The study site was near the village of Malesina on the east coast of Central Greece approximately 120 km north of Athens (38° 35'N, 23° 15' E). This is the same locality as that

described for the outplanting experiment in Chapter 5. A large wildfire occurred there in August 2000, burning several thousands of hectares of *Pinus halepensis* forest. In November 2000 a total of five different burned forest stands were selected from within the wider burned landscape. These five stands were labelled A-E.

The stands were located in a variety of slope and aspect combinations but were similar in species composition and forest physiognomy. Within each stand, three plots measuring 50 m x 50 m were established. Stand characteristics were recorded at one randomly chosen location per plot. These locations corresponded to one of the resprouting shrub microsites selected in the experimental design (see below). At each of these the following variables were recorded: slope, aspect, tree density, tree DBH, maximum tree height (Carl Leiss altimeter). Tree density was estimated from the number of trees occurring within a 10 m radius of the selected shrub. Stand characteristics are shown in Table 6.1. At each of Stands C, D and E each of the three plots were located on alternating spurs on opposite sides of a meandering stream such that the aspect of the three plots differed from one another by as much as 180° (Table 6.1).

Percentage cover of rocks was estimated at three randomly selected resprouting shrubs per plot. At each of these locations a three-metre tape was laid on the ground surface along a random compass bearing and the total length of intersection with rocks or stones greater than 1 cm was recorded.

### 6.2.2 Experimental design

A schematic representation of the sampling design is presented in Figure 6.1 and detailed below. First seed germination occurred at the beginning of November. At the end of November, seedlings of *Cistus creticus* located at three different microsite types were selected in each of the three plots in each stand. The microsite types were the same as in Chapter 3, i.e, within 30 cm of an ectomycorrhizal shrub ["EM-shrub"] (*Quercus coccifera*), a non-ectomycorrhizal shrub ["Non-EM shrub"] (*Pistacia lentiscus*) or in open areas at least 1 m from any resprouting shrub. "EM-shrub", "Non-EM-shrub" and "Open" are henceforth collectively referred to as "microsites"

Within each plot, candidate shrubs and open areas (those with seedlings) were identified and three representatives of each microsite type were randomly selected and the seedlings associated with them were tagged. Tags consisted of flags constructed from short lengths of metal wire and red Matsel tape placed in the soil next to the seedlings to avoid damaging their fragile stems.

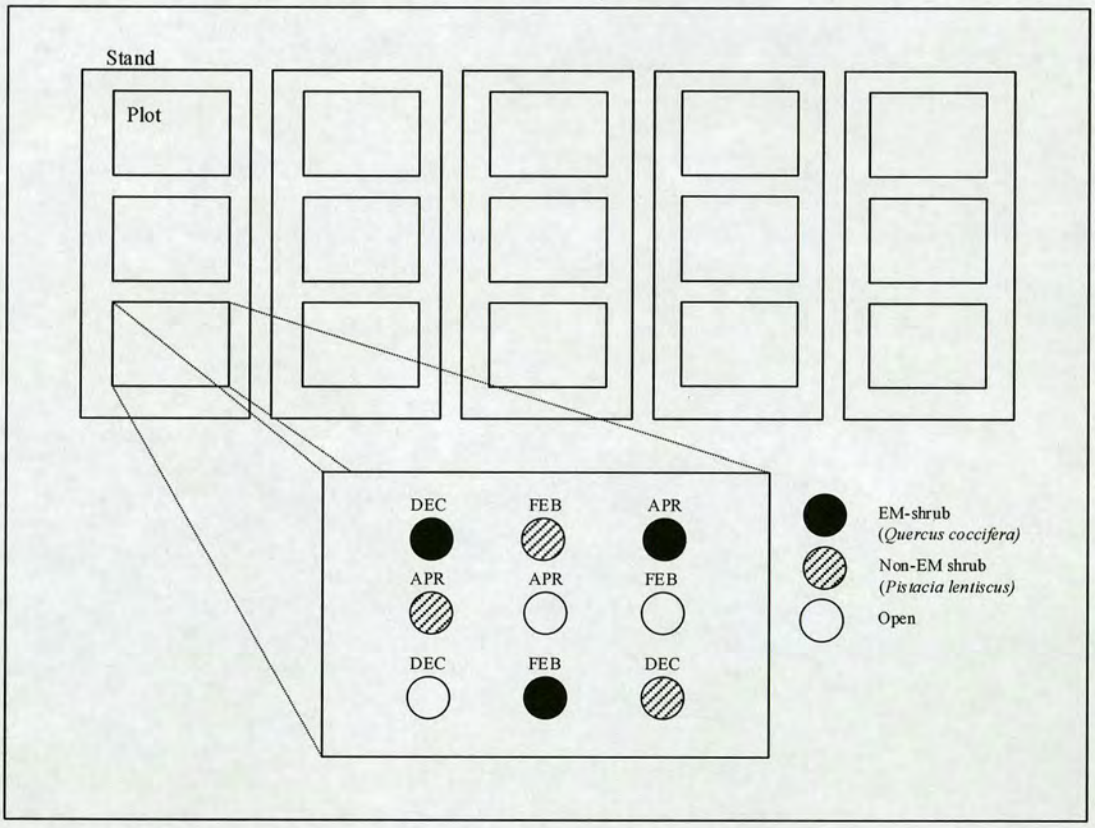
At the time of tagging the number of true leaves and shoot height (root collar to apical tip) were recorded. Primary leaves and secondary leaves were recorded separately and are referred to as "Leaves1" and "Leaves2" respectively (see Chapter 5, Figure 5.1). These measurements indicated seedling size at the start of the experiment (variable = StartHt).

Table 6.1. Stand characteristics. Values are means (±s.e.) of the three plot values except for aspect for which the individual values for the three plots are presented. Soil pH, P, K and Mg values are the overall means of samples collected at all microsite types and at all harvest times (n = 27 for each stand). * Missing data.

| | Stand A | B | C | D | E |
|---|---|---|---|---|---|
| Slope (degrees) | 29 ± 1 | Flat | 15 ± 1 | 9 ± 0 | 10 ± 1 |
| Aspect (degrees) | 300 | Flat | 50-190-50 | 90-90-260 | 90-240-40 |
| % cover rocks | 37.6 ± 5.3 | 7.2 ± 3.6 | 10.6 ± 1.1 | 21.0 ± 3.8 | 32.4 ± 6.5 |
| Tree density (No./ha)(<9.5 cm DBH) | 541 ± 205 | 361 ± 112 | 191 ± 110 | 329 ± 219 | 233 ± 53 |
| Tree density (No./ha)(>9.5 cm DBH) | 393 ± 77 | 350 ± 80 | 509 ± 18 | 393 ± 56 | 329 ± 28 |
| Mean DBH (cm) (trees >9.5 cm DBH) | 13.2 ± 0.3 | 15.6 ± 0.5 | 17.5 ± 0.6 | 23.3 ± 2.5 | 22.9 ± 1.5 |
| Tree height (m) | * * | 14.0 ± 0.6 | 16.7 ± 0.7 | 15.5 ± 0.4 | 14.0 ± 0.6 |
| pH | 8.3 ± 0.01 | 7.8 ± 0.05 | 8.1 ± 0.02 | 8.1 ± 0.03 | 7.9 ± 0.04 |
| P (mg/l) | 2.9 ± 0.6 | 1.9 ± 0.4 | 3.5 ± 0.7 | 4.6 ± 0.7 | 4.3 ± 1.1 |
| K (mg/l) | 399.0 ± 21.7 | 520.0 ± 19.2 | 549.8 ± 20.3 | 560.1 ± 26.1 | 578.8 ± 21.5 |
| Mg (mg/l) | 1341.9 ± 26.8 | 393.4 ± 15.2 | 304.7 ± 13.3 | 366.7 ± 19.0 | 344.9 ± 13.9 |

The selected microsites were randomly assigned to each of three harvest times (December, February, April). December, February and April are henceforth collectively referred to as "harvests". One harvest every other month was felt to be adequate to monitor temporal changes in the EM fungal community and also was the maximum effort affordable in the allotted time. The total number of seedlings tagged was 135 (5 stands x 3 plots x 3 microsite types x 3 harvests) comprising 15 replicates of each microsite type at each harvest (5 stands x 3 plots).

Figure 6.1 Schematic diagram of the experimental design for sampling naturally establishing *Cistus creticus* seedlings in burned forest stands in Central Greece. Harvest times (DEC, FEB, APR) were randomly assigned to seedlings in each plot.




### 6.2.3 Harvest

Seedlings were harvested at three times: December (2-8) 2000 (DEC), February (13-17) 2001 (FEB), April (7-12) 2001 (APR). Since first seed germination occurred at these sites at the beginning of November the harvests took place at intervals of one, three and five months respectively after germination. At each harvest all relevant tagged seedlings were measured as at the start to record their growth increment during the experiment. Distance of seedling to

nearest resprouting stem (StemDist) and to nearest burned pine tree trunk (PineDist) were recorded for all seedlings being harvested at that time. In addition, the number of resprouting stems and height of tallest stem were recorded at the shrub microsites. Excavated seedlings were wrapped in moist kitchen towel, sealed in plastic bags and placed in a cool box for transport back to the laboratory. A soil sample was collected from every sampling point. Soil immediately adjacent to the excavation of each seedling was removed to a depth of 10 cm using a trowel and sealed in a plastic bag.

### 6.2.4 Sample processing

Soil samples were placed in foil dishes and allowed to air-dry for one week. They were then sieved to 4 mm, crushed with mortar and pestle, sieved to 2 mm and placed in self-seal plastic bags for transport back to Scotland. Soil samples were analysed by the Scottish Agricultural College Analytical Services Department for pH (calcium chloride suspension), phosphorus (Olsen's method), potassium and magnesium (extraction with modified Morgan's solution).

Seedling shoots were removed at the root collar, placed in paper envelopes and oven dried at 80°C for 48 hrs. The envelopes were then placed in self-seal plastic bags and stored at 4 °C. The root systems were carefully washed free of soil under running tap water and placed in water in Petri dishes. They were then further cleaned manually with the aid of a dissecting microscope and stored at 4 °C in 2% glutaraldehyde until assessed.

### 6.2.5 Assessment of mycorrhizas

***a) Short roots***

Entire root systems of all seedlings were examined intact in Petri dishes in water under a dissecting microscope. All root tips were recorded as non-mycorrhizal or according to ectomycorrhizal morphotype. The same three measures of colonization described in Chapter 4 (section 4.2.7a) were calculated for each morphotype: percentage colonization (percentage of total root tips colonized), relative abundance (i.e., percentage of colonized root tips) and frequency of occurrence.

***b) Long roots***

For the December harvest, seedlings from one of each of the microsites per stand were randomly selected for assessment of long-root colonization. All seedlings harvested in

February and April were assessed. Thus 15 seedlings were assessed in December and 45 seedlings in both February and April.

After voucher specimens of ectomycorrhizal short-root tips had been removed, the remaining root system of each seedling was cut into fragments of approximately 1 cm length and placed in modified syringes for clearing and staining (see Chapter 2, section 2.4.3 for details of clearing and staining methodology). Stained roots were laid parallel to one another in PVLG on microscope slides and examined at ×200 and ×400 magnification. Long root colonization was quantified using the method described in Chapter 4 (section 4.2.7b). Long root colonization is also referred to as percentage of root length colonized.

### 6.2.6 Statistical analysis

*a) Multivariate analyses*

In order to test for the effect of microsite and harvest-time on ectomycorrhizal community structure, the data were analysed by ordination using the Canonical Correspondence Analysis (CCA) function in the computer program CANOCO (Ter Braak & Smilauer, 1997-2002). To avoid pseudoreplication in the statistical analysis, data were pooled across plots within stands. Pooling of morphotype data was achieved by summing the number of root tips recorded for each morphotype and for non-mycorrhizal root tips across plots for each microsite × harvest combination. This was to avoid zero values of percentage colonization contributing to the test values and also avoided the problems of an unbalanced design caused by missing values. Environmental data were pooled by taking the arithmetic mean across plots for each microsite × harvest combination.

The strategy for analysis was to analyse the effect of microsite and harvest separately. Thus for the analysis of microsite, "EM-shrub", "Non-EM-shrub" and "Open" were entered as nominal environmental variables. Harvest, stand, soil pH, phosphorus, potassium and magnesium, as well as height at the start of the experiment (StartHt) and the height increment during the experiment (dHt) were entered as covariables in order to isolate the effect of microsite. Biplot scaling and inter-sample distances were used. Data were log transformed (Log(x + 1)) and downweighting of rare species was applied. Monte Carlo permutation testing was carried out with 999 permutations under a full model restricted to blocks defined by combinations of harvest and stand. The analysis was repeated to test for the effect of harvest with December, February and April replacing microsites as environmental variables and "EM-shrub", "Non-EM-shrub" and "Open" replacing harvests as covariables.

The relationship between samples, morphotypes and soil chemistry was investigated at the level of individual seedlings using CCA. In order to test the significance of this relationship it was necessary to constrain the Monte Carlo permutations to independent samples within blocks defined by combinations of harvest and stand. Split-plot permutation testing within CANOCO requires balanced experimental design. Unfortunately due to missing data the final design was unbalanced. To achieve a balanced design, samples from one plot in each stand were removed. This left a total of 90 seedlings for the analysis (5 stands x 2 plots x 3 harvests x 3 microsites). The raw data were percentage of total root tips colonized. Prior to ordination data were arcsine square-root transformed and downweighting of rare species was applied. Soil pH, log(phosphorus), potassium and magnesium were entered as environmental variables. Harvest, microsite and stand, as well as seedling height at the start of the experiment (StartHt) and the height increment during the experiment (dHt) were entered as covariables. Monte Carlo permutation testing was carried out with 999 permutations under a full model restricted to blocks defined by harvest and stand.

***b) Univariate analyses***

Diversity indices were calculated as follows:

1. Margalef's species richness:

$$d = (S\text{-}1)/\mathrm{Log}(N)$$

where $S$ is the number of morphotypes in a sample
and $N$ the total number of individuals (root tips) in a sample.

2. Shannon-Wiener diversity:

$$H' = -\Sigma\,(P_i \,.\, \mathrm{Ln}(P_i))$$

where $P_i$ is the proportion of individuals that the $i$th species contributes to the total number of individuals in the sample.

3. Simpson's Dominance index:

$$SI = \Sigma\,(Pi^2).$$

The effects of microsite and harvest-time on soil chemistry, diversity indices, percentage colonization and seedling growth were analysed using two-way, mixed-model, nested ANOVA. Plots and stands were considered as random factors with plots nested within

stands. Microsite and harvest-time were considered as fixed factors. The main effects F-ratios for microsite and harvest-time were derived from the sum of squares of the respective two-way interactions with 'Stand' where this interaction was significant. Where the interaction with 'Stand' was not significant, main effects F-ratios were derived after pooling the sums of squares and degrees of freedom of the interaction and error terms. The F-ratio for the interaction between harvest and microsite was derived from the three-way interaction between stand, microsite and harvest where this was significant and by pooling sums of squares and degrees of freedom with the error term where it was not.

Analyses were carried out on the untransformed data and the residuals checked for normality. Data with non-normal residuals were transformed ($Log_{10}(x+1)$ or arcsine($\sqrt{(x/100)}$)), re-analysed and the residuals checked again. Homogeneity of variance was checked with Bartlett's test. Due to seedling mortality the design was unbalanced and the data were therefore analysed using the Generalised Linear Model routine in MINITAB. The structure of the ANOVA and degrees of freedom for each source of variation are given in Table 6.2.

Table 6.2 ANOVA structure and degrees of freedom (df) for parameters analysed. A – Soil chemistry (pH, P, K, Mg). B – Species richness (Margalef) and Shannon-Wiener diversity. C – Simpson's Dominance index. D – percentage of root tips colonized. E – Percentage of root length colonized. F – Number of primary leaves. G – Number of secondary leaves, height.

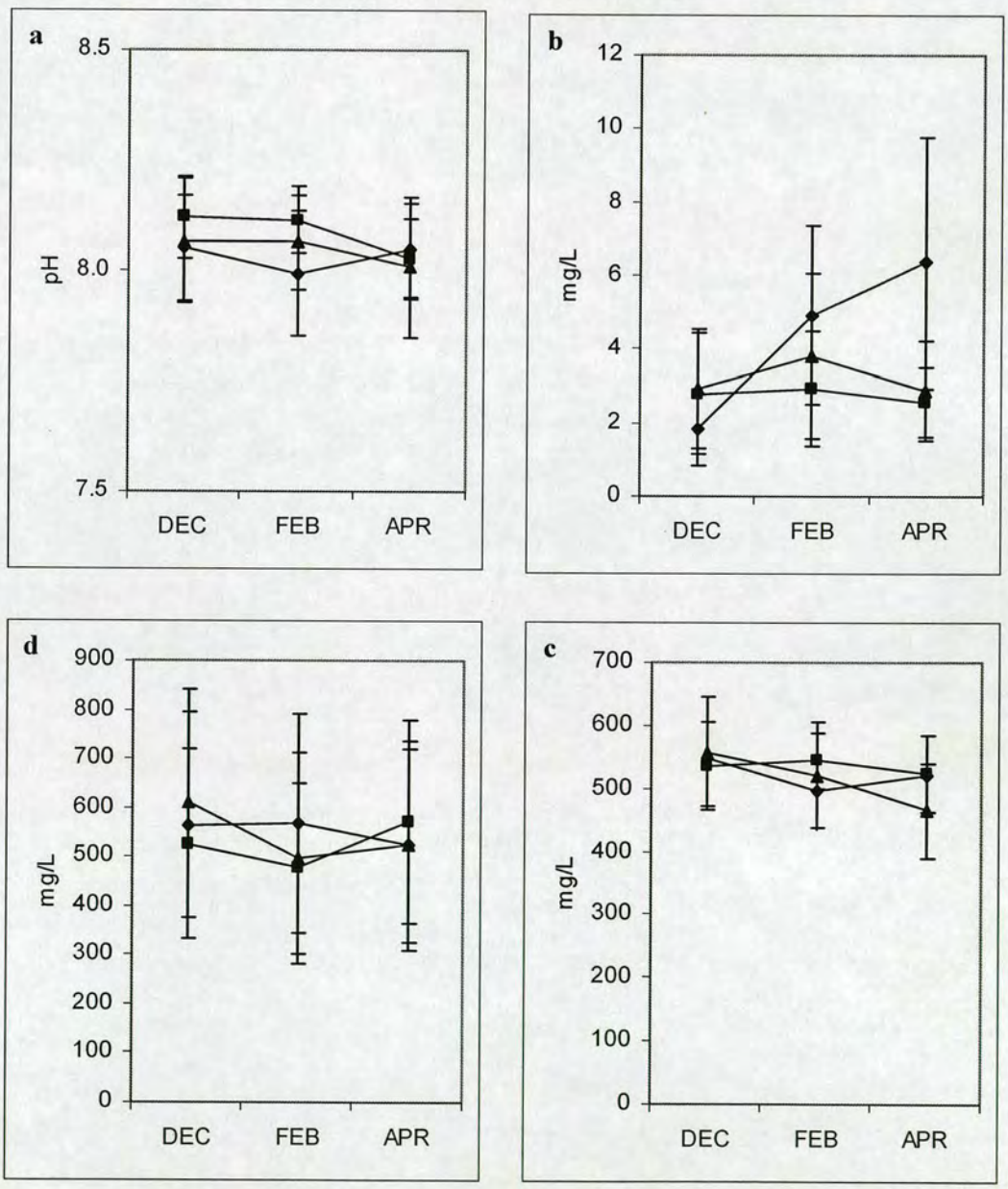
| Source | Analysis | | | | | | |
|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G |
| Stand | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Plot (Stand) | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Microsite | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Stand x Microsite | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| Harvest | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Stand x Harvest | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| Microsite x Harvest | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Stand x Microsite x Harvest | 16 | 16 | 16 | 16 | 16 | 16 | 16 |
| Error | 77 | 70 | 69 | 76 | 49 | 73 | 74 |
| Total | 131 | 124 | 123 | 130 | 103 | 127 | 128 |

## 6.3 Results

### 6.3.1 Soil chemistry

There was a significant interaction between microsite and harvest-time for soil phosphorus ($F_{4,[16+77]} = 2.71$, $P = 0.035$). P increased with time around *Quercus coccifera* shrubs but not *Pistacia lentiscus* shrubs or in open areas (Figure 6.2). This trend meant that by April, soil phosphorus was higher around *Quercus* shrubs compared to the other microsites. Soil pH, potassium and magnesium did not vary significantly with either microsite or harvest-time (Figure 6.2). However all of the soil chemistry parameters varied significantly between stands (pH, $F_{4,10} = 11.3$, $P = 0.004$; K, $F_{4,10} = 8.0$, $P = 0.038$; Mg, $F_{4,10} = 276.0$, $P = 0.008$). Stand A was slightly low in potassium and extremely high in magnesium compared to the other stands (Table 6.1).

Figure 6.2 Effect of microsite and time on a) soil pH, b) phosphorus, c) potassium and d) magnesium in burned *Pinus halepensis* stands in central Greece. ♦ = EM-shrub, ў = non-EM-shrub, ▲ = Open. Error bars are 95% confidence intervals.



### 6.3.2 Ectomycorrhizal fungal community

***a) Effect of harvest and microsite***

A total of 17 morphotypes was recorded. Canonical correspondence analysis revealed a significant effect of harvest but not of microsite on ectomycorrhizal community structure (Table 6.3). The effect of harvest is illustrated in Figure 6.3. The correlations between the first CCA axis and December, February and April harvests were 0.6175, -0.3583, and -0.3867 respectively, indicating that the effect of harvest was driven by the dissimilarity of the December samples to the February and April samples.

The effect of harvest on the frequency and abundance of individual morphotypes is shown in Figure 6.4. The EM communities at the February and April harvests differ from that at the December harvest mainly in the addition of several morphotypes (Ascomycete 2, Unknown 12, Unknown 11, *Genea*-like, Unknown 2, *Tuber* 3). Overall, three groups of fungi can be defined.

1. Fungi common or frequent soon after burning and declining with time (Unknown 1, Basidiomycete 3, *Thelephora terrestris*)

2. Fungi frequent to rare or absent soon after burning and increasing with time (E-Strain, Unknown 4, *Tuber* 1, Ascomycete 2, Unknown 12, *Genea*-like, Unknown 2, *Tuber* 3)

3. Fungi not changing in frequency and abundance with time. These may be frequent (Ascomycete 1) or rare (*Cenococcum geophilum*, Thelephoroid 2). This group probably also includes the rare morphotypes picked up in less than 5% of samples (*Tuber* 2, *Inocybe* 2, Unknown 11) where recorded absence from particular harvests is likely to be accounted for by sampling error.

Of the total colonized root tips recorded from the burned stands the level of dominance of the three commonest morphotypes was broadly similar at each harvest time (Figure 6.5). However, the identity of those dominant types differed between harvests. In December, 73% of the total number of colonised root tips sampled was attributed to morphotypes Unknown 1, Ascomycete 1 and Basidiomycete 3. In February, 73% was attributed to Unknown 1, *Tuber* 1 and Unknown 4 and in April, 75% was attributed to Unknown 2, E-Strain and Unknown 4.

Although the CCA ordination suggested that there was no significant influence ($P = 0.081$) of microsite on community structure with respect to relative abundance of morphotypes,

analysis of total species richness showed a significant interaction between microsite and harvest-time ($F_{4,16} = 3.38, P = 0.033$) (Figure 6.6).

Table 6.3 Results of canonical correspondence analysis (CCA) of effect of microsite and harvest on ectomycorrhizal community structure.

| | Microsite | Harvest |
|---|---|---|
| Total inertia | 1.483 | 1.483 |
| Sum of all eigenvalues | 0.872 | 0.878 |
| Sum of all canonical eigenvalues | 0.056 | 0.094 |
| % of sample variation explained by treatment | 6.4 | 10.7 |
| **Monte Carlo test** | | |
| 1st canonical axis | $F = 1.80, P = 0.081$ | $F = 2.26, P = 0.047$ |
| all canonical axes | $F = 1.06, P = 0.287$ | $F = 1.80, P = 0.029$ |

Figure 6.3 Ordination diagram of Canonical Correspondence Analysis (CCA) of the ectomycorrhizal community associated with *Cistus creticus* seedlings establishing in burned *Pinus halepensis* forest stands. Seedlings were harvested at three times: December, February, April following a forest fire in August. Samples are grouped by harvest with harvest centroids represented by filled triangles.

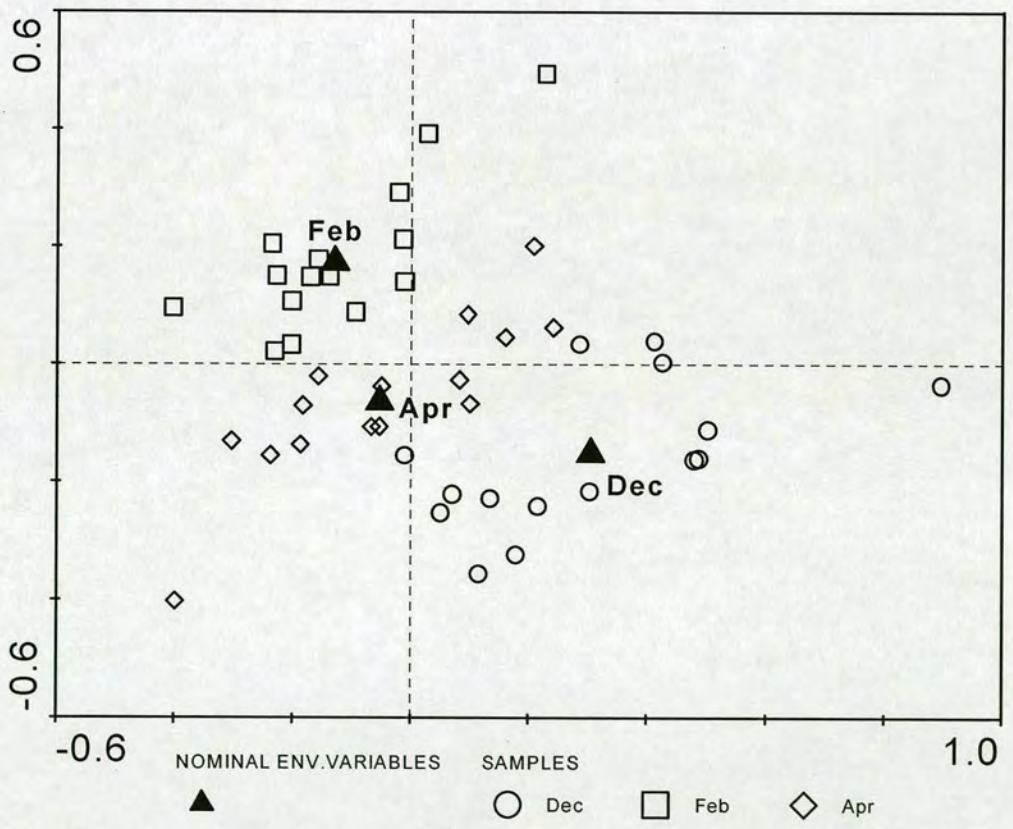


Figure 6.4 Effect of harvest time on frequency (solid bars), relative abundance (open bars) and percentage colonization (hatched bars) of ectomycorrhizal morphotypes colonizing *Cistus creticus* seedlings establishing in burned forest areas in central Greece. Error bars = standard error where n > 2.

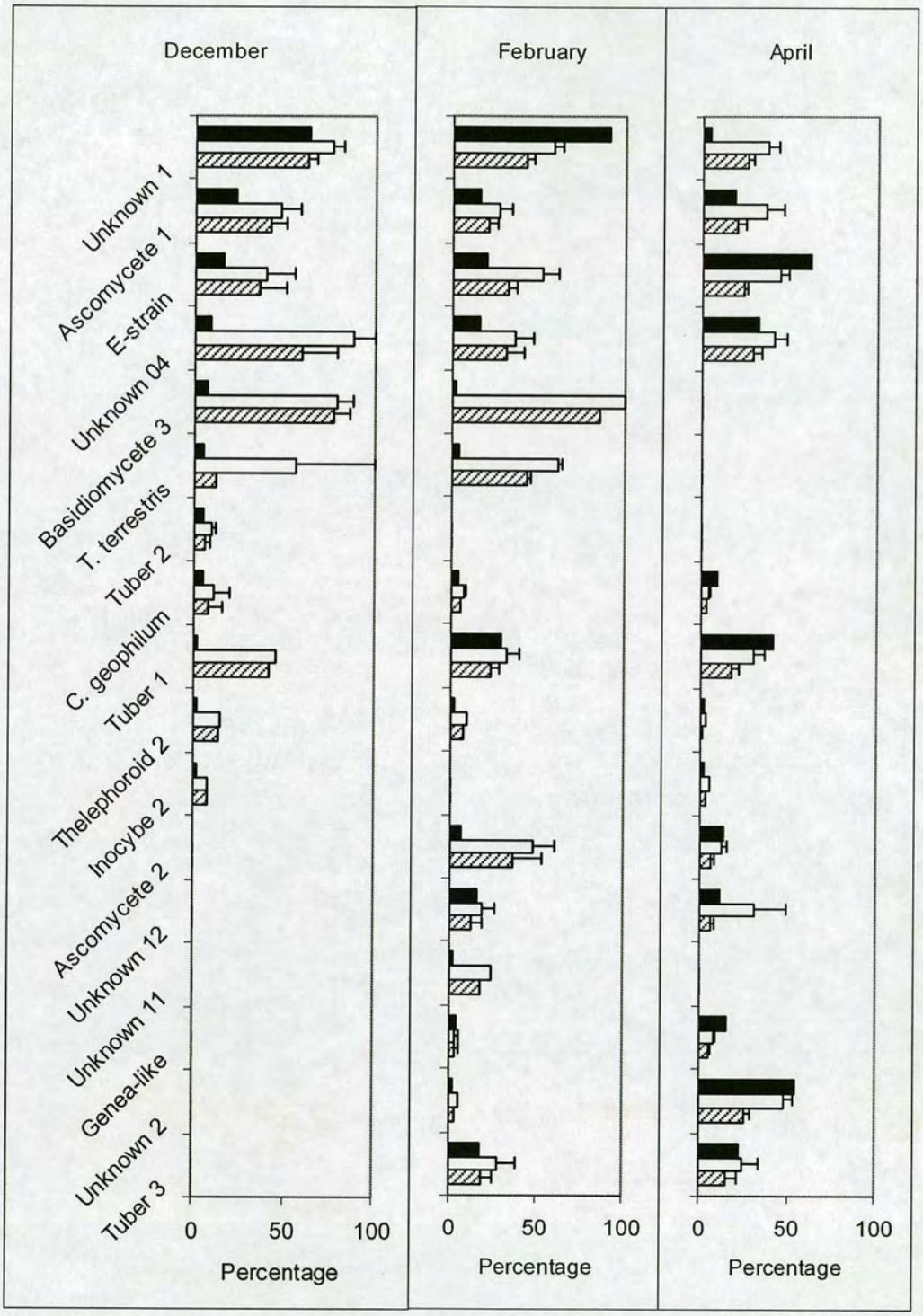
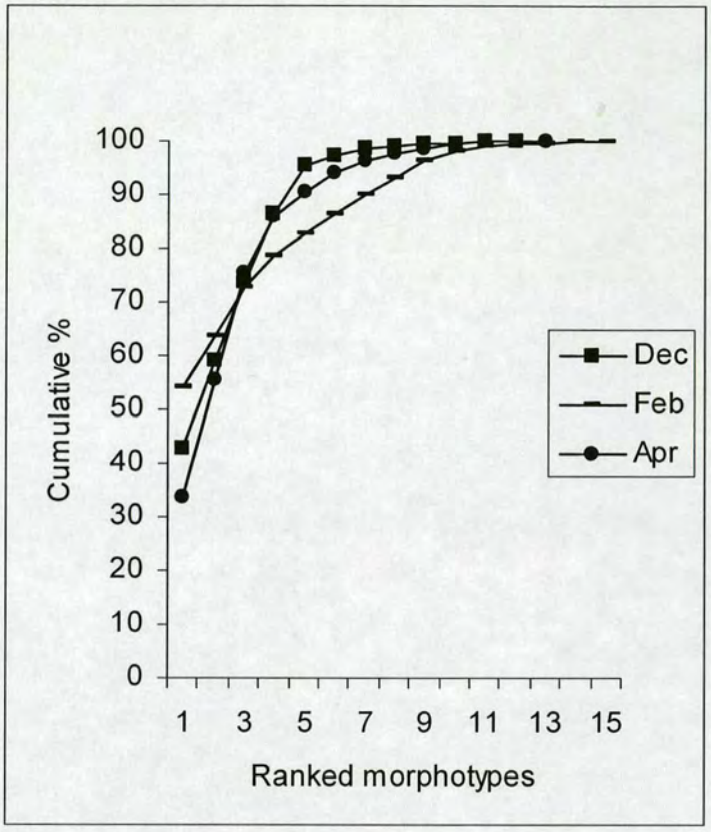


Figure 6.5 K-dominance plots for number of root tips colonized by ectomycorrhizal fungi in *Cistus creticus* seedlings establishing in burned forest areas in central Greece.

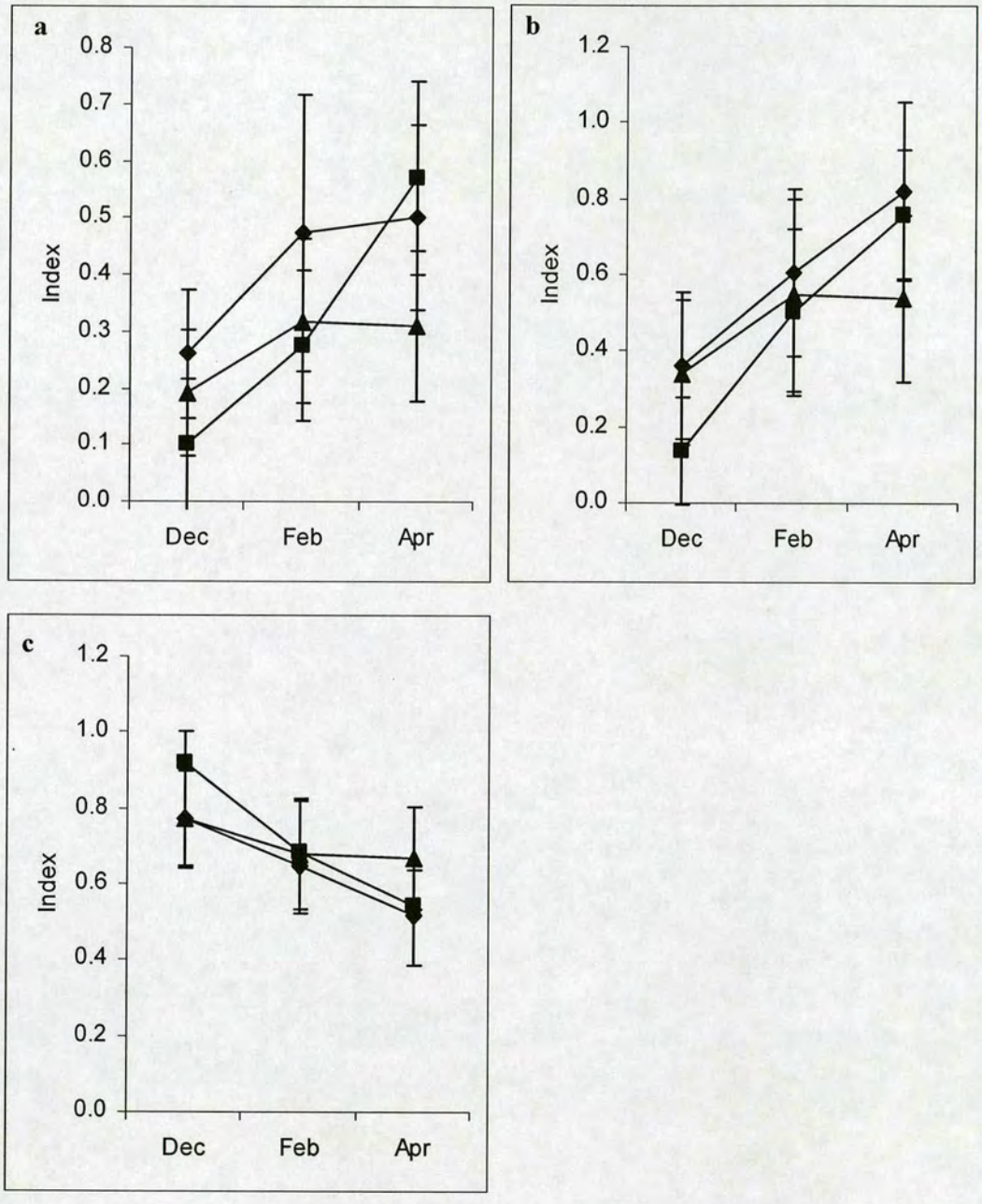



By April, species richness around *Quercus* and *Pistacia* shrubs had increased relative to open areas (Figure 6.6). This pattern was also reflected in the Shannon-Wiener and Simpson's Dominance indices though the interaction was not significant for these variables (Figure 6.6). There was a significant main effect of harvest-time on species richness ($F_{2,8}$ = 5.92, $P$ = 0.03), Shannon-Wiener diversity ($F_{2,8}$ = 4.50, $P$ = 0.05). However, the effect of harvest-time varied significantly with stand for both indices.

***b) Relationship between morphotypes and soil chemistry***

Canonical correspondence analysis showed weak and insignificant ($P$ = 0.70) relationships between the soil chemistry variables and ectomycorrhizal community structure among samples. Within the constrained ordination space, these environmental variables together accounted for only 4.3% of the total variance in the morphotype data.

Figure 6.6 Effect of microsite and time on a) species richness (Margalef), b) Shannon-Wiener diversity and c) Simpson's Dominance Index for ectomycorrhizal fungi associated with *Cistus creticus* seedlings establishing in burned forest areas in central Greece. ♦ = EM-shrub, ŷ = non-EM-shrub, ▲ = Open. Error bars are 95% confidence intervals.

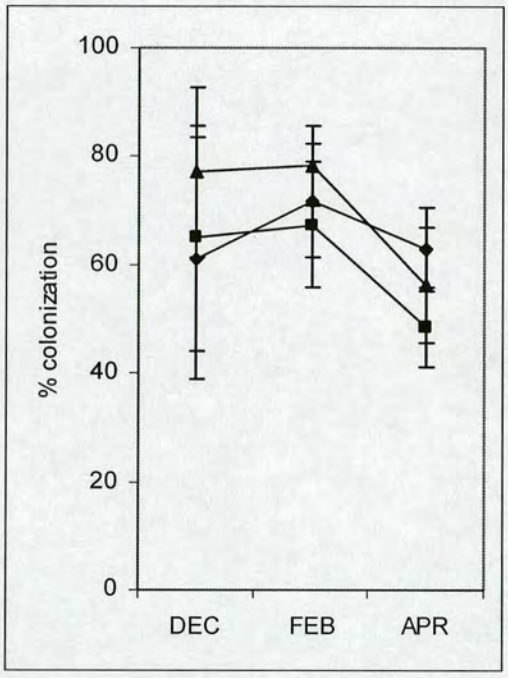


### 6.3.3 Mycorrhizal colonization

*a) Short roots*

Mean percentage of root tips colonized was significantly affected by harvest-time ($F_{2,[8+76]}$ = 4.0, $P$ = 0.02). Colonization was high in December and February (60-80%), particularly in open areas, and then decreased in April at all microsites (50-60%) (Figure 6.7).

Figure 6.7 Effect of microsite and time of harvest on percentage of root tips colonized of *Cistus creticus* seedlings establishing in burned forest plots in central Greece. ♦ = EM-shrub, ŷ = non-EM-shrub, ▲ = Open. Error bars are 95% confidence intervals.



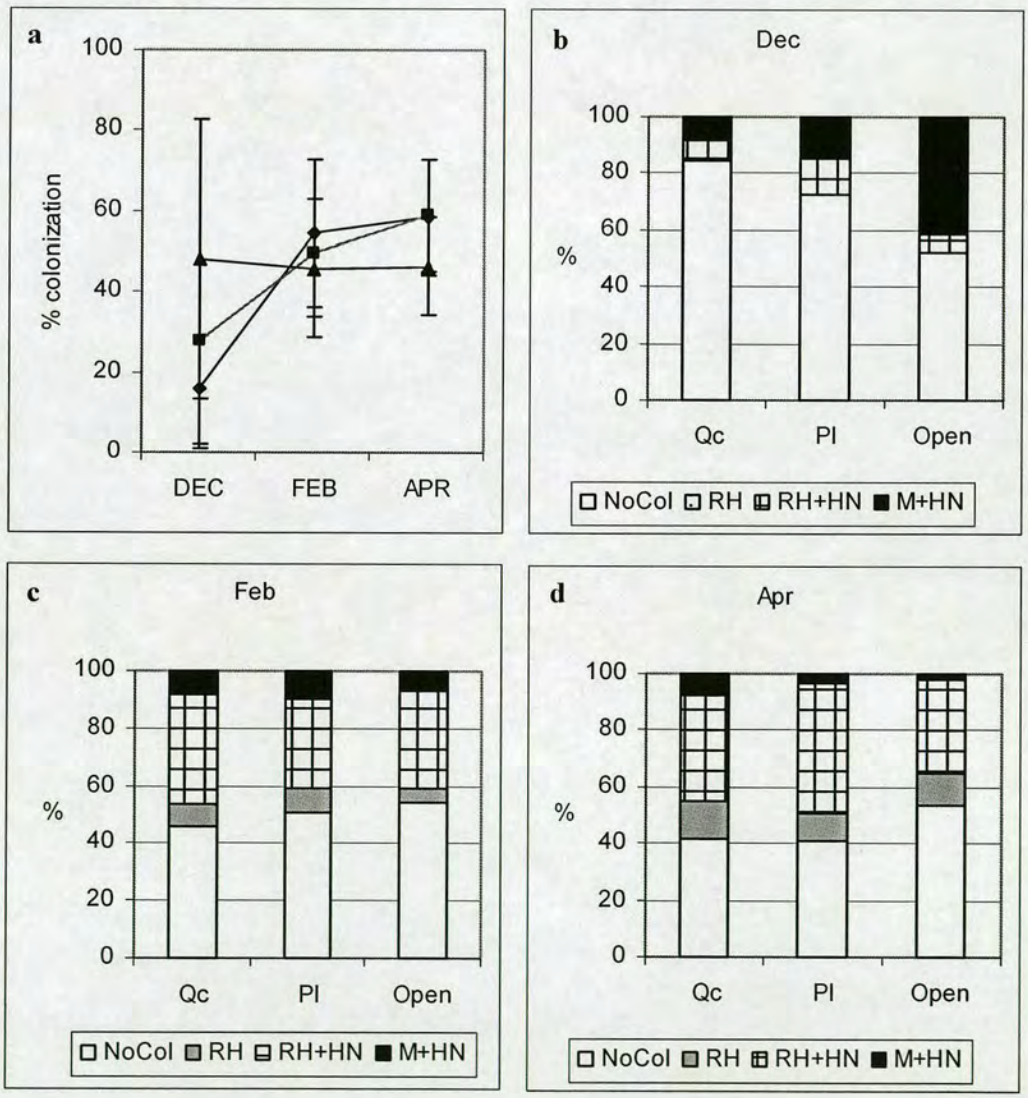

*b) Long roots*

The same pattern of long-root colonization observed in the greenhouse bioassay of forest soils and there attributed to morphotype Unknown 1 (Chapter 4), was recorded in these naturally establishing seedlings. The hyphal characteristics and patchy, Hartig-net-like internal structures associated with long root colonization appeared to be the same in both cases. Hyphae occurring on the root surface were 6-10 μm diameter, smooth-walled, unmelanised and irregular in outline. The same cytoplasmic disjunctions occurred in both cases. These were characterised by cytoplasm on one side of a septum appearing granular

and with many inclusions that stained darkly in cotton blue while on the other side it was markedly less granular and without inclusions.

The levels of long-root colonization were variable between samples, particularly at the first harvest, and were not significantly affected by microsite or harvest-time (Figure 6.8a). The trend was towards low colonization close to *Quercus* and *Pistacia* shrubs in December, rising to maximal levels of 50-60% root length colonized in February and April. In the open areas, similar maximal levels were already recorded in December and maintained through February and April.

Figure 6.8 Effect of microsite and time of harvest on percentage of lateral root length colonized. a) Total percentage root length colonized. ♦ = EM-shrub, ŷ = non-EM-shrub, ▲ = Open. Error bars are 95% confidence intervals. Figures b)-d) show the proportion of the total root length colonized accounted for by the four colonization categories at the December, February and April harvests respectively. NoCol = no colonization, RH = running hyphae only, RH+HN = running hyphae + Hartig net, M+HN = Mantle + Hartig net.



In the open areas in December the majority of the colonization of long roots was attributed to the highest fungal load class, involving coalescence of hyphae on the root surface to form a mantle and the presence of a Hartig net (Figure 6.8b). By February, the majority of colonization at all microsites was attributed to fungal load class 3 (running hyphae on the root surface and presence of Hartig net) (Figure 6.8c, d). The proportion of colonization where only running hyphae were recorded was always relatively small indicating that where there was interaction between these hyphae and long roots, Hartig net was nearly always formed.

### 6.3.4 Seedling growth

Microsite had no effect on any of the growth parameters measured for *Cistus creticus* seedlings during the first six months of establishment in the burned forest (Figure 6.9). As expected, harvest-time had a significant effect on number of primary leaves ($F_{2,[8+73]} = 435.8$, $P < 0.001$), number of secondary leaves ($F_{2,8} = 103.2$, $P < 0.001$) and shoot height ($F_{2,8} = 94.8$, $P < 0.001$). However, the latter two parameters varied significantly between stands.

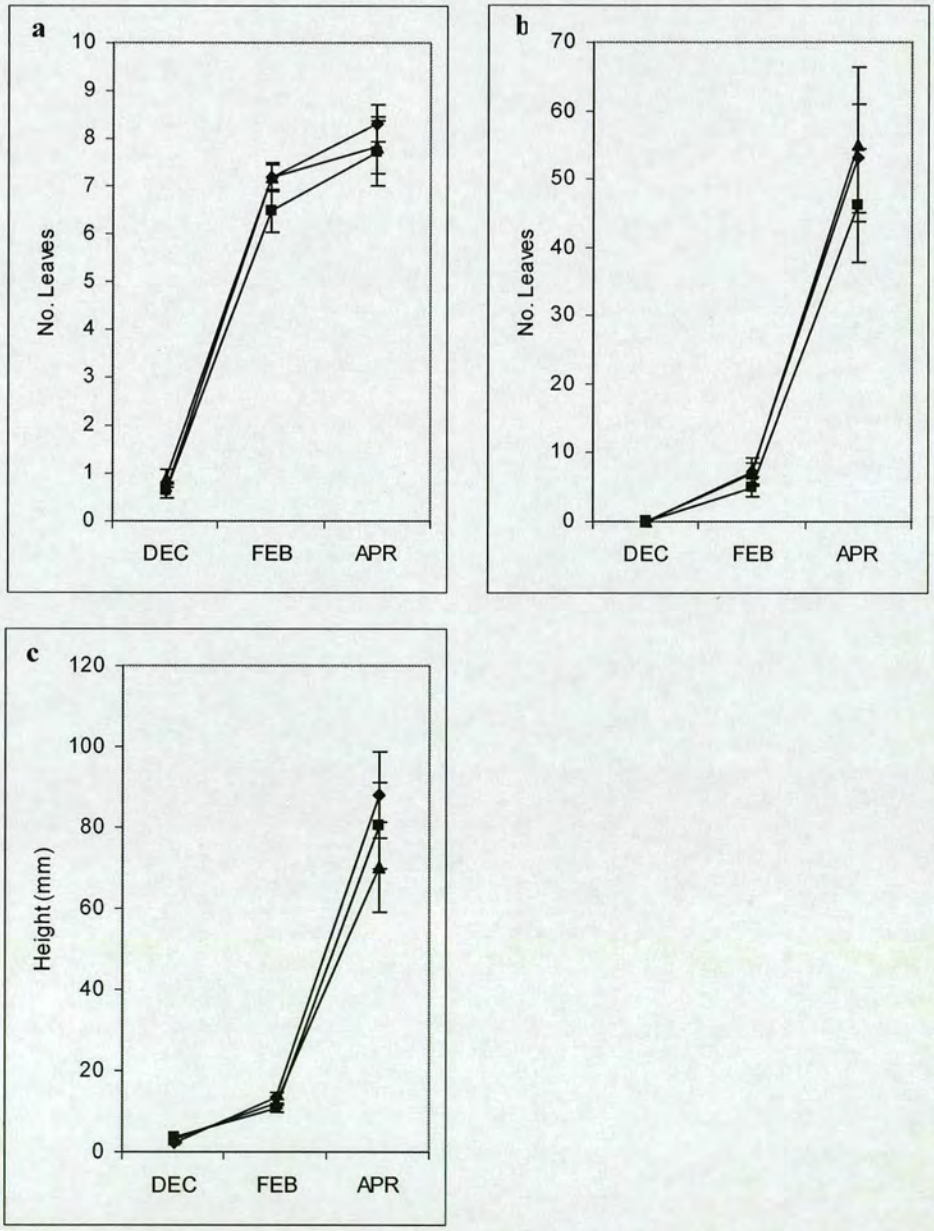
Most of the seedling productivity during December and January was invested in the production of primary leaves (Figure 6.9a). From February to April, investment was switched from primary to secondary leaves and the height increment increased sharply (Figure 6.9a, b, c).

## 6.4 Discussion

The results show that *Cistus* seedlings form ectomycorrhizas in the first month after germination. Indeed, even at this early stage of development the overall average percentage of root tips colonized was 68% and included a fairly broad range of fungi (11 morphotypes recorded in the December harvest). Early mutualistic association with soil fungi may be a strategy that has evolved to enable *Cistus* to maintain small seeds through rapid provision of external nutrients. It is unclear what the natural pressures selecting for small seed size in *Cistus* are. *Cistus* regenerates after fires from a soil seed bank that accumulates during the fire interval. It is generally considered that *Cistus* persists in the forest that develops after fire for only approximately fifteen years and then declines as more vigorous understorey shrubs such as *Quercus coccifera and Pistacia lentiscus* crowd it out (Arianoutsou, 1998; Roy, 1992). As the fire return period is generally much greater than 15 years, the seed bank must necessarily persist for a long time. Studies have shown that there is an inverse relationship between seed size and persistence in the seed bank (Thompson *et al.*, 1998; Thompson *et al.*,

1993). Small compact seeds are more likely to become buried and therefore protected from the effects of both predation and fire. Furthermore, as *Cistus* seedlings establish after fires that remove aboveground biomass, there is no selective pressure for large seed size associated with establishment under conditions of low light intensity or high competition. Therefore, by circumventing the disadvantages of small seed size associated with poor nutrient provisioning, ectomycorrhizal fungi may allow *Cistus* to derive the benefits of small seed size associated with persistence.

Figure 6.9 Effect of microsite and time of harvest on growth of *Cistus creticus* seedlings establishing in burned forest areas in central Greece. a) Increment in number of primary leaves. b) Increment in number of secondary leaves. c) Increment in shoot height. ♦ = EM-shrub, ŷ = non-EM-shrub, ▲ = Open. Bars are standard errors.



Earliest colonization of short roots was dominated by the morphotype Unknown 1 that was both frequent among seedlings and abundant relative to other morphotypes. As well as colonizing short roots, hyphae associated with the morphotype Unknown 1 were also found colonizing the long roots in the same way as observed in the greenhouse bioassay (Chapter 4). Here again, but this time in nature, long-root colonization was characterised by hyphae running along the root surface, coalescing in places to form mantle-like structures and also running through the interstitial spaces within the root cortex forming Hartig-net-like structures around some cells. Long-root colonization was somewhat variable in extent in the first month after germination but occupied an average of 30% of lateral root length in individual seedlings. In this first month, all long-root colonization involved formation of Hartig-net-like structures and the majority was characterised by coalescence of hyphae on the root surface to form a mantle-like structure. Interestingly, both long-root colonization (Figure 6.8) and short-root colonization (Figure 6.7) tended to be higher in the open microsites in December compared to the two shrub microsites though the high level of variation rendered this effect statistically insignificant. It is unclear why this might be. None of the soil chemistry parameters measured in the present study varied much between microsites at the December harvest. It is possible that other soil parameters not measured here do vary between the microsites and influence the level of colonization. Further studies examining a wider range of soil parameters are required.

By February, the average percentage of root length colonized had risen to 50% and was maintained at this level in April. However, by February and into April, the proportion of long root with a mantle-like structure had declined and the majority of colonization was characterised by the presence of running hyphae and Hartig net. Other studies have recorded early colonization of pine seedling long roots by ectomycorrhizal fungi (Sohn, 1981; Wilcox, 1968). These studies recorded inverse relationships between the growth of long roots and the extent of colonization. Mantle and Hartig net development was most sparse where root growth rate was highest. Thus while formation of a mantle on sections of long root appears to be a prominent feature during the early developmental stages, increasing lateral root growth rate soon outstrips the rate at which the fungi can form continuous mycorrhizal structures.

Increasing root growth rates probably also explain the decrease in percentage of root tips colonized. It appears that as the seedlings grew, the rate at which new root tips were produced became greater than the rate of colonization by EM fungi. Increase in long root colonization in the form of running hyphae and discontinuous Hartig net and decrease in root

tip colonization with time were also observed in the greenhouse bioassay (Chapter 4) suggesting a degree of ecological relevance to these observations.

While long root colonization by Unknown 1 increased with time, the frequency of seedlings with colonization of short roots by Unknown 1 had declined dramatically by the April harvest. This was accompanied by an overall increase in morphotype diversity. These results suggest that Unknown 1 is a rapid and prolific colonizer of seedlings immediately after seed germination but it is soon replaced on the short root tips by other fungi while maintaining its position in the long roots. This may represent a form of niche partitioning that enables Unknown 1 to co-exist on the roots of *Cistus* with other, more dominant ectomycorrhizal fungi. Further studies are required to assess the extent to which long root colonization by Unknown 1 is maintained as the host matures.

Multivariate analysis suggested that the EM fungal community changed during the first five months after post-fire seedling establishment. In addition to the apparent replacement of Unknown 1 by other fungi on the short roots, there were identifiable differences between morphotypes in their response to fire. Comparison of Figures 5.5 (Chapter 5) and 6.4 shows that nine of the morphotypes recorded at the December harvest in the present study were recorded from seedlings planted into unburned forest plots in the same area (Ascomycete 1, E-strain, Unknown 4, *Thelephora terrestris*, *Tuber* 1, Thelephoraceae 2, *Inocybe* 2, Unknown 13, *Genea*-like). Unknown 4 was very frequent in the unburned forest but infrequent in the December harvest suggesting that it was initially severely affected by the fire. However, it had increased in frequency by February and increased further by April to a level similar to that recorded from the burned plots in the outplanting experiment that were also assessed in April. Thus, Unknown 4 is characterised by initial post-fire depression but rapid recovery. In contrast to this, the early-stage fungi *Thelephora terrestris* and *Inocybe* 2 both appeared to be reduced in frequency after the fire but slow to recover. This is consistent with the hypothesis put forward in Chapter 5 that these early-stage fungi are susceptible to fire and slow to recover because their spores are not fire resistant and they exist at low mycelial abundance in the unburned forest where they are out-competed for available root space by other fungi. They probably survive by exploiting small-scale local disturbances to competing mycelia that might arise from the digging activities of small mammals and the burrowing of soil invertebrates. This implies a high turnover of small patches of mycelium of early-stage fungi in mature forests. Such a pattern is supported by recent molecular studies of fungal genet size and persistence inferred from analysis of sporocarp DNA in *Laccaria amethystina* (Fiore-Donno & Martin, 2001; Gherbi *et al.*, 1999) and *Hebeloma cylindrosporum* (Gryta *et al.*, 1997). In the latter species correspondence between above- and

belowground patterns were observed (Guidot *et al.*, 2001). However, similar patterns of genet distribution have also been reported for several late-stage fungi in mature forests (Bergemann & Miller, 2002; Redecker *et al.*, 2001; Zhou *et al.*, 1999) which have also been related to belowground patterns of root colonization (Zhou & Hogetsu, 2002). Evidently there is much still to be learned about how ectomycorrhizal fungal communities are structured in mature forests.

Although in general microsite appeared to have little effect on community structure, the increase in total species richness with time was greater at the shrub microsites compared to open areas. The four morphotypes positively identified as Basidiomycetes by the presence of clamp connections, *Thelephora terrestris*, *Inocybe* 2, Thelephoroid 2 and Basidiomycete 2, together were present in a total of 13 out of the 135 seedlings excavated. Ten of these were from shrub microsites. This again provides some evidence, albeit weak and circumstantial, of shrub microsites acting as sources of post-fire recolonization by EM basidiomycete fungi as seen in Chapter 3. The question remains as to whether this possible increase in colonizing potential of basidiomycetes around resprouting shrubs is stimulated by an enhanced resource base associated with shrub root production or whether it is due to an effect of the soil conditions on the fungi. The only real microsite effect on soil conditions recorded here was a significant increase in phosphorus around *Quercus* shrubs from December to April that was not recorded at the other microsites. However, at the seedling level there was no apparent relationship between EM community structure and the soil chemistry parameters measured. Again, seedling level effects may be detected for other soil parameters not measured here. It is also possible that microsite effects on soil structure and chemistry that impinge on fungal growth may be felt more strongly in sub-surface soil layers.

# Chapter 7 – General discussion

## 7.1 Summary of findings

Results presented in the previous chapters suggest that the earliest post-fire colonization of *Cistus* seedlings throughout the forest is dominated by E-strain fungi and an unidentified fungus (Unknown 1) that form weak ectomycorrhizas in the upper portions of root systems. Early colonization of long roots in addition to short roots appears to be an important process in one of these fungi (Unknown 1). Unknown 1 is subsequently replaced on short roots by a wide range of mature forest fungi indicating that it may be a poor competitor. E-strain fungi, on the other hand appear able to persist, at least for the first six months of seedling establishment. The weak ectomycorrhiza-forming fungi probably colonize from spores or other resistant propagules while the mature forest fungi colonize from mycelia that are attached to an existing resource-base that may be either living or dying roots of mature forest plants.

In general, resprouting shrubs had little quantifiable effect on percentage colonization or ectomycorrhizal community structure though there was some circumstantial evidence to suggest that some mature forest fungi, particularly basidiomycetes are restricted to soils around resprouting shrubs in the early stages of post-fire recovery. Rather, by providing new roots for the fungi to colonize, it is the *Cistus* seedlings themselves that facilitate the post-fire recovery of mature forest fungi. Thus the *Cistus* seedlings seem to form a vital component of the forest cycle that ensures maintenance of the ectomycorrhizal fungal community after fires. Early colonization of *Cistus* by weak ectomycorrhiza-forming fungi may facilitate the establishment of the *Cistus* seedlings thus forming a further link in the cycle.

## 7.2 Early colonization by weak ectomycorrhiza-forming fungi

Fungi that form loose ectomycorrhizal associations, i.e, with mantles that are thin and discontinuous to virtually absent, are conspicuous features of the early colonization of *Cistus* seedlings. *Cistus* seed is very small and apparently lacking in nitrogen and potassium (Hanley & Fenner, 1997) and establishing *Cistus* seedlings therefore have an immediate requirement for external sources of these nutrients. Early association with mycorrhizal fungi may represent a strategy that has evolved in response to this requirement. However, the amount of photoassimilate produced by very young seedlings is unlikely to be able to

support fungi with a high carbon demand. Such fungi require a supply of carbon from larger resource bases associated with living or perhaps dying roots of mature plants. These fungi appear to be largely excluded from the top few centimetres of burned soils in the first months after fires and are largely unavailable to establishing seedlings when they are producing their first roots. Therefore it may be an advantage to the seedlings to be able to form associations with fungi that are present in the upper soil layers as long as the carbon demand of those fungi is small.

For fungi to exert a small carbon demand on their hosts but at the same time also be able to forage for nutrients away from the immediate rhizosphere they may require additional, alternative supplies of carbon from elsewhere. It is possible that these fungi may be deriving additional energy by the oxidation of carbon compounds assimilated saprotrophically from soil organic matter. Limited saprotrophy among ectomycorrhizal fungi under laboratory conditions is not a new concept. Many ectomycorrhizal fungi are culturable on agar and therefore evidently have the capacity to live in isolation from living plant hosts when supplied with simple sugars. Some ectomycorrhizal fungi are known to produce pectinolytic and proteolytic enzymes suggesting that they also have the potential to metabolise more complex organic molecules (Abuzinadah *et al.*, 1986; Dahm & Strzelczyk, 1995; Dahm *et al.*, 1999). However, true saprotrophy would require the production of cellulase and other enzymes capable of directly breaking down complex carbohydrates in recalcitrant litter. Several studies have demonstrated that ectomycorhizal fungi do possess such enzymes (Colpaert & van Laere, 1996; Dahm & Strzelczyk, 1995) though their activity is evidently low compared to decomposer fungi (Colpaert & van Laere, 1996).

It remains to be discovered to what extent and under what conditions ectomycorrhizal fungi can function saprotrophically in nature. The pertinent question concerning supplementary saprotrophy in weak ectomycorrhiza forming fungi is whether fungi are able to function saprotrophically in one part of a mycelium and biotrophically in another part of the same mycelium. Such functioning would require fine control of expression of degradative enzymes or perhaps inhibition of those enzymes by the host supporting that part of the mycelium living biotrophically. Laboratory experiments have shown that some ectomycorrhizal fungi can assimilate nutrients from complex organic sources and transfer these to their host plants (Perez-Moreno & Read, 2000). This suggests that fungi are able to localize the expression of degradative enzymes within a single mycelium.

A simpler and more traditional explanation for the carbon economy of weak ectomycorrhiza-forming fungi is that their carbon demand is lower and adequately supplied by even the youngest host seedlings. Whatever the case with the fungi, young seedlings are likely to

benefit from the low drain on their carbon resources. Where the extent of colonization by ectomycorrhizal fungi is limited to the formation of Hartig net without the development of thick hyphal sheaths surrounding the roots, seedlings may benefit from exchange of nutrients without having to support a large hyphal mass. This type of colonization was particularly apparent in the morphotype Unknown 1 that was such an important component of the early colonization of *Cistus* seedlings.

## 7.3 Identity of Unknown 1

The identity of Unknown 1 remains uncertain. I originally thought that species of post-fire ascomycetes might be responsible. Fruiting bodies of *Anthracobia* cf. *macrocystis* became conspicuously abundant soon after the first rains after summer fires in the forest sites in Greece, at the same time that *Cistus* seedlings were establishing. Species of *Anthracobia* have been reported to form rudimentary ectomycorrhizas *in vitro* with *Pinus contorta* seedlings (Egger & Paden, 1986b). Furthermore, a fungus identified as a species of *Geopyxis* was isolated from an ectomycorrhizal root tip of a pine seedling establishing after a fire (Chapter 2). *Geopyxis carbonaria* has recently been demonstrated to form ectomycorrhizas with mature *Picea abies* (Vralstad *et al.*, 1998). The obvious question was whether these post-fire ascomycete fungi form a mycorrhizal association with establishing *Cistus* seedlings. The results of the synthesis experiment suggested that they do not (Chapter 2). No ectomycorrhizal structures were formed. Furthermore the morphology of the hyphae of Unknown 1 did not appear similar to any of the three post-fire ascomycetes tested. It is therefore likely that the species of *Geopyxis* isolated from the pine root was merely present as an inhabitant of the rhizosphere feeding on pine root exudates and this may be the case for other post-fire ascomycetes. However, it is possible that the experimental conditions were not ideal for the formation of ectomycorrhizal structures. Further synthesis experiments with *Cistus* seedlings and post-fire ascomycetes are required in which the experimental conditions more closely mimic those found after fires in nature.

Another proposition as to the identity of Unknown 1 put forward in Chapter 2 was that it is formed by species of *Tuber*. *Tuber* normally forms fully developed ectomycorrhizas with *Cistus* and it was suggested that the incomplete formation of ectomycorrhizal structures seen in Unknown 1 may be due to colonization by monokaryotic *Tuber* strains. If this were the case it is necessary to ask why *Tuber* is forming fully developed ectomycorrhizas with naturally establishing seedlings in the sub-surface post-fire soil layers (Chapter 3) and forming incomplete ectomycorrhizas in the upper post-fire soil layers and in bioassay. It may be that root tips in the sub-surface soil layers are colonized by surviving dikaryotic

mycelium while the partially colonized root tips in the top few centimetres are the result of colonization by monokaryotic hyphae soon after spore germination. The ubiquity of Unknown 1 in burned (Chapters 3, 4, 5 and 6) and unburned (Chapter 4) soils suggests that the propagules responsible must be widely dispersed before the fire and able to survive a heat shock. However, *Tuber* species tend to form truffles at some depth beneath the soil surface thus requiring trained dogs or pigs to find them. Spores are released into the soil when the truffle decomposes or are deposited on the soil surface in the faeces of animals that have eaten these nutritious fruitbodies. In the former case most spores would be concentrated beneath the soil surface and therefore are not likely to be responsible for the widespread formation of Unknown 1 in the upper centimeters. In the latter case, we might expect spores to be deposited in a much more patchy distribution though the possibility of redistribution by wind and water after deposition cannot be ruled out. Interestingly it has recently been proposed that *Tuber* species exhibit a saprotrophic phase in their life-cycle (Giovannetti *et al.*, 1994). Some time after formation of the carpophore from primordia initiated by hyphae emanating from ectomycorrhizas, the connecting hyphae die and the further development of the carpophore relies on the saprotrophic capabilities of newly formed hyphae. This suggests a degree of nutritional versatility in *Tuber* that requires further investigation.

The affinity between Unknown 1 and the desert truffles *Terfezia* and *Tirmania* was also presented in Chapter 2. Observations on the ecology of desert truffles coupled with the results of the present study regarding the spatial distribution of Unknown 1 lend support to an affinity with them. Heat resistance in spores of *Terfezia* and *Tirmania* species remains untested. However, unlike *Tuber* these species are known to form truffle-type fruitbodies near the soil surface, actually breaking the surface of the ground as they mature (Awamah & Alsheikh, 1978; Taylor *et al.*, 1995). This would suggest that spores of these species become concentrated in the upper soil layers of the unburned forest. There may therefore be a selective pressure for heat resistance of spores in these species and it would be interesting to test this and compare with *Tuber*. Furthermore, both monokaryotic and dikaryotic mycelia of *Terfezia* and *Tirmania* species are known to colonize roots and form mantle-less mycorrhizas (Giovannetti *et al.*, 1994). This would allow immediate colonization of roots after spore germination by monokaryotic mycelia. Early access to a source of readily assimilable carbon may be beneficial to the fungus in its early stages of growth after fires in the harsh environment of the ash and upper soil layer. Indeed, laboratory experiments have demonstrated that addition of activated charcoal to the growth medium stimulates rapid and extensive formation of typical desert truffle ectomycorrhizas (Hartig net but partial or absent mantle) between *Terfezia leonis* and *Helianthemum sessiliflorum* (Roth-Bejerano *et al.*, 1990). To explain this it was proposed that adsorption of nutrients onto charcoal particles

created a nutrient gradient between fungus and root that elicited a growth response of the fungus towards the root.

Synthesising this information it is possible to propose the following scenario. After the fire *Terfezia/Tirmania* spores lie in the upper soil layers immediately below the ash layer. After the first rains in October, *Cistus* seeds germinate and quickly begin to produce roots in the upper soil layers. *Terfezia/Tirmania* spores also germinate. Seeking nutrition the new hyphae grow towards *Cistus* roots along a nutrient gradient created by adsorption of nutrient ions to charcoal particles in the ash layer. Hyphae colonize all parts of the developing root system, forming Hartig net-like structures in both short and long roots. As the root system penetrates deeper into the soil it encounters inocula of mature forest fungi including *Tuber* spp. and full ectomycorrhizal root tips are formed. As the seedling continues to grow, the desert truffles are replaced on the short roots by recovering mature forest fungi but remain in the long roots to some extent.

The proposal of this scenario is speculative and requires further testing. In particular, the identity of Unknown 1 remains a crucial and so far, missing piece of information. A combination of molecular and traditional synthesis techniques should be employed to address this.

The above scenario emphasises the dynamic nature of the post-fire fungal community and in particular the probable importance of competitive interactions between ectomycorrhizal fungi in determining observed patterns of colonization.

## 7.4 Competition in EM fungi

Though Unknown 1 was an important early colonizer of *Cistus* seedlings, it appeared to be sensitive to competition from other EM fungi. It commonly formed associations with root tips in the upper portions of root systems in recently burned forest where there were few other fungi (Chapter 3). It was less common in the lower portions of those root systems where recovering mycelia of mature forest fungi were more abundant. Unknown 1 also completely dominated the roots of seedlings grown in greenhouse bioassays of burned and unburned soil where few other fungi were recorded. It did not colonize short roots in field bioassays of unburned forest plots (Chapter 5) and appeared to be largely displaced from the short roots of naturally establishing seedlings by other EM fungi by the fifth month after post-fire seed germination (Chapter 6).

Competition between fungi may result from either or both of two basic processes: primary resource capture and direct combat (Cooke & Rayner, 1984). The former results in the exclusion of one fungus by another through superior abilities in nutrient utilization or colonization of space (in the form of commonly utilizable substrate) thus denying access to these resources. Direct combat between fungi arises when mycelia of two incompatible strains come in contact and a form of chemical warfare is initiated in which the outcome is either stalemate or the replacement of one mycelium by another. Little research has been directed towards understanding competitive interactions between mycorrhizal fungi.

Some evidence of direct competitive replacement in ectomycorrhizal fungi has been provided by Wu *et al.* (1999) who observed the overgrowth and replacement of *Pisolithus tinctorius* ectomycorrhizas by another, unidentified ectomycorrhizal fungus in experimental rhizoboxes. Evidence of competitive exclusion can be seen in the ability of EM fungi such as *Laccaria bicolor* to limit colonization by naturally occurring fungi when inoculated onto seedlings and outplanted in forestry plantations (Selosse *et al.*, 1998; Villeneuve *et al.*, 1991). Competitive exclusion has also been observed between *Tuber melanosporum* and some species of ectomycorrhizal greenhouse contaminants on pot-grown hazel plants (Mamoun & Olivier, 1996; Olivier & Mamoun, 1994). In that case, the outcome of competitive interactions between the different fungi varied according to competitor identity and host root volume. *Tuber melanosporum* was able to co-occur with an unidentified species of basidiomycete but was excluded by the ascomycete *Pulvinula globifera* (Olivier & Mamoun, 1994). When host root volume was small there was generally a mutual exclusion of symbionts (Mamoun & Olivier, 1996). When host root volume was large, co-occurrence occurred, presumably because the different fungi were able to colonize separate parts of the root system. On the other hand, when *Pinus pinea* seedlings were inoculated with *Tuber borchii* together with *Laccaria bicolor, Hebeloma sinapizans* and *Sphaerosporella brunnea* and transplanted into forest plantation sites, the *Laccaria, Hebeloma* and *Sphaerosporella* inoculants were completely replaced by *T. borchii* and indigenous fungi within the planting site (Zambonelli *et al.*, 2000). These studies indicate that the outcome of competitive interactions between ectomycorrhizal fungi are species dependent.

In the present case it is probable that both competitive replacement and competitive exclusion are occurring. In the sequential harvest of naturally establishing seedlings, the frequency of Unknown 1 declined dramatically between the first and fifth months after *Cistus* germination while the richness of associated species increased (Chapter 6). This suggests that Unknown 1 was replaced by other fungi. Certainly the lack of a proper mantle in this morphotype probably contributes to its susceptibility to replacement. Hyphae of

competing fungi coming into contact with colonized short roots are not challenged by hyphae already occupying the root surface as in the case of fungi that form morphotypes with mantles. The absence of Unknown 1 from the unburned plots of the outplanting experiment (Chapter 5) is more likely to be the result of competitive exclusion by other fungi as the duration of the experiment was probably too short for Unknown 1 to have colonized roots and for all those roots to have been replaced by other fungi.

In addition to competitive interactions between ectomycorrhizal fungi, it is becoming increasingly clear that ectomycorrhizal and saprotrophic fungi compete with one another for the nutrients that are locked up in litter (Leake *et al.*, 2002). As saprotrophic fungi are likely to be even more localised within the forest litter layer, the removal of this by wildfire should significantly reduce this source of competition. This may be another factor contributing to the observed proliferation of Unknown 1 in the burned plots.

Competitive ability in EM fungi is a function of physiological traits but is perhaps only one aspect of the colonizing potential of a fungus. Others include inoculum type, inoculum abundance and abiotic soil characteristics. Together these contribute to the overall inoculum potential at any given point in the soil which in turn determines the level of colonization of an establishing seedling.

## 7.5 Variation in level of colonization

The level of colonization among individual seedlings was often highly variable with samples harbouring a single morphotype ranging from less than 10% to 100% colonized (Chapter 3). The lack of correlation between level of colonization and number of leaves or seedling height suggests that this variation was not determined by the developmental state of the seedlings at this early stage of establishment. As the seedlings grow larger, they seem to exert a greater influence on the level of colonization. This was seen particularly in the decline in percentage of root tips colonized by the fifth month after germination (Chapter 6). By this time it appears that the production of new roots is proceeding at a rate faster than the rate of colonization by ectomycorrhizal fungi. It is likely that after several years, as litter accumulates and mature forest fungi recover, levels of colonization would increase again. However, in the first few months after germination the level of colonization must be a function largely of soil inoculum potential at the site of establishment.

Undoubtedly there is variation between fungi in the level to which they colonize seedlings. Fungi such as Unknown 1 and E-Strain occur frequently after fires but tend to colonize relatively moderate proportions of seedlings' root systems. By contrast, some fungi such as

Basidiomycete 3 and *Thelephora terrestris* occur infrequently but where they do occur, occupy large proportions of root systems (see Figure 6.4). This is probably related to differences in fungal physiology and growth rates but may perhaps also be influenced by the degree of receptivity of the plant to particular fungi. Related to inoculum identity is type of inoculum. Whether a fungus is colonizing from spores or from intact mycelia that are connected to and being supplied with carbon from living mature plants will affect the rate and extent of root colonization. The amount of fungal inoculum available in post-fire soils will also affect rate and extent of colonization.

Inoculum identity, type and amount are all features of the fungi involved. The context in which those fungi occur may also influence the outcome of seedling colonization. In particular heterogeneity in soil nutrients and organic matter may affect the way that fungi respond to plants and vice versa.

## 7.6 Fungal response to the abiotic environment

It has been demonstrated numerous times under controlled laboratory conditions that mycorrhizal association between plants and fungi is stimulated under conditions of low soil nutrients (Smith & Read, 1997). To date little effort has been directed towards testing this relationship in nature (Erland & Taylor, 2002). The present study was able to demonstrate few convincing correlations between percentage colonization of short or long roots and any of the soil chemical parameters measured (Chapter 6). However, due to limited time and resources only pH, phosphorus, potassium and magnesium were measured. There are many more parameters that could exert an influence on mycorrhizal colonization. I believe that it may be worthwhile pursuing the question of abiotic influence on mycorrhizal colonization in terms of fungal behaviour and response to the external environment.

It is generally considered that the relationship between levels of soil nutrients and levels of root colonization by mycorrhizal fungi is controlled principally by the host plant (Smith & Read, 1997). In instances where the fungi involved are obligately biotrophic, as in the case of arbuscular mycorrhizal fungi, this is undoubtedly the case. However, where many of the fungi can apparently live independently of their hosts, at least to some degree, controlling mechanisms are not so clear-cut and fungal responses to the abiotic environment may be more important.

Nutritional versatility has long been recognised as a characteristic of the fungi with many species apparently able to switch between saprotrophic, necrotrophic and biotrophic life-styles (Cooke & Rayner, 1984). At the level of the individual, fungal behaviour within a

particular nutritional mode may be influenced by fungal responses to the abiotic environment and in particular the balance between external and internal fungal resources. A framework for understanding the response of fungal individuals to the external environment has recently been proposed whereby the degree of 'openness' of fungal boundaries is the key parameter (Rayner *et al.*, 1999). Where abiotic stress is low fungi are presented with an unrestrictive environment that favours the development of an exploitative life-style (Rayner *et al.*, 1999). This is characterised by diffuse mycelial organization plus high assimilation and proliferation rates. Where stress aggravation occurs the environment becomes restrictive and fungi may switch towards more coherent mycelial organisations in the form of resistant structures such as sclerotia, and chlamydospores that facilitate survival. Such structures are characterised by the sealing of dissipative mycelial boundaries through hyphal fusion resulting in conservation of internal resources (Rayner *et al.*, 1999).

For mycorrhizal fungi that exhibit some degree of nutritional versatility the extent to which they form associations with plants may in part be influenced by the restrictiveness of the extra-radical environment for those fungi. Where the soil environment is restrictive, the formation of mycorrhizal structures may serve to reduce the dissipation of fungal resources. In mycorrhizas, which are defined by a bi-directional transfer of nutrients between fungus and host, dissipative fungal boundaries can only be partially sealed. However, loss of nutrient resources to the host plant is balanced by gain of carbon resources and may be low compared to other parts of the mycelium that inhabit the wider soil environment.

## 7.7 Mature forest fungi and resprouting shrubs

While the early colonization of seedlings by weakly ectomycorrhizal fungi is apparently a frequent and probably functionally important process it does appear to represent a measure that bridges the initial depression of the colonizing potential of the mature forest fungi.

The results presented here suggest that the mature forest fungi are depressed by the action of fire but that they persist beneath the surface of the soil. The patchiness of their distribution is likely to be related to the distribution of the roots of the forest plants on which they were established before the fire. Where these roots are located close to sites of new root production, for example the ligno-tubers of ectomycorrhizal resprouting shrubs, the opportunity to establish new ectomycorrhizas and thereby connect with a fresh supply of carbohydrates would enhance the colonizing potential of these fungi.

The evidence for a refugial effect of resprouting shrubs remains rather equivocal. In the spatial analysis of inoculum distribution (Chapter 3), the majority of Basidiomycete species

were only recorded on seedlings growing in close proximity to *Quercus coccifera* or *Pistacia lentiscus* shrubs. They did not colonize the seedlings excavated from gap microsites between the shrubs. However in the subsequent experiments this pattern was not so clearly observed. Basidiomycete fungi were observed on seedlings from open microsites in burned plots in both the field bioassay and the sequential harvest albeit infrequently. The lack of strong patterning in inoculum associated with shrub microsites in these studies may reflect the lack of patterning in the distribution of plant roots.

The fresh supplies of carbohydrate required for mature forest fungi to recover are likely to come largely from the *Cistus* seedlings themselves. The amount of carbon that can be supplied by an individual seedling is likely to be small. However, after fires *Cistus* seedlings often form quite dense swathes carpeting the forest floor. A mycelium of an ectomycorrhizal fungus could easily colonize groups of seedlings establishing in close proximity to one another thereby receiving carbon from several seedlings together.

## 7.8 Critique and future research

### 7.8.1 General

While the present study has been broadly successful in documenting, for the first time, the course of early post-fire colonization of *Cistus creticus* seedlings by mycorrhizal fungi, some aspects of the research require refinement in order to understand better the dynamics of this process.

The most obvious weakness of the present study, and therefore requirement for future research, is the unknown identity of most of the morphotypes described. Molecular techniques are required to address this problem. Analysis of Restriction Fragment Length Polymorphism (RFLP) in the Internal Transcribed Spacer (ITS) region of nuclear ribosomal DNA has so far proven to be a robust method for the identification of ectomycorrhizas (Gardes & Bruns, 1993; Gardes *et al.*, 1991; Kårén *et al.*, 1997). The method relies on the ability to match ITS-RFLP patterns from mycorrhizal roots with those from material of known identity. Ideally this would be fresh sporocarps collected from the same sites at which root material has been collected. However, given the ephemeral and irregular nature of fungal sporocarp production and the general paucity of specimens found in Aleppo pine forests during the course of this investigation, recourse would need to be made to the extraction of DNA from herbarium material (Bruns *et al.*, 1990). In the absence of positive

RFLP matches, the ITS region can be directly sequenced and compared against sequences deposited in databases such as GenBank.

As discussed above, particular attention should be given to identifying the fungus that is forming morphotype Unknown 1 and colonizing substantial portions of the long roots. In addition, experiments are required to assess the functional characteristics of this association in order to determine the extent to which it is nutritionally beneficial to the young *Cistus* seedlings. Of further interest is the effect of soil conditions, particularly the presence of charcoal particles in ash deposits, on the behaviour of this and other fungi. The post-fire environment provides conditions of heterogeneous soil nutrients conducive to the study of the influence of the extra-radical soil conditions on the dynamics of mycorrhiza formation.

*Cistus* is an excellent model plant for studying these questions. Its small seed size ensures that it has an immediate requirement for external sources of nutrition and thus may provide a mycorrhizal system that is particularly sensitive to nutrient heterogeneity. *Cistus* has further advantages in being easy to propagate and fast growing thus facilitating experimental work. It is also abundant in nature so that the ecological impact of research is relatively trivial.

The present study has focussed exclusively on the mycorrhizal associations of *Cistus* seedlings. To better understand the dynamics of fungal recovery after fires research is required to investigate the root associations of the mature plants in both the burned and the unburned forest. Particular attention should be paid to characterising the ectomycorrhizal associations of resprouting shrubs such as *Quercus coccifera*. This will not be easy as soil coring is virtually impossible in the shallow, hard and rocky soils that are characteristic of the areas where *Q. coccifera* grows. Instead, heavy equipment may need to be employed to excavate trenches adjacent to shrubs and then roots recovered by careful hand excavations of the trench face. Similar attention should be paid to *Arbutus* shrubs as these also have the potential to act as refugia from which ectomycorrhizal fungi may colonize establishing *Cistus* seedlings.

*Pinus halepensis* is also stimulated to seed after fires and it would be interesting to compare the dynamics of ectomycorrhizal colonization of the seedlings of this species. Although not reported here, some work was done on pine seedlings. These observations suggest that pine seedlings only start to form ectomycorrhizas in the third month after germination rather than immediately as in the case of *Cistus*.

### 7.8.2 Methodologies

Observations of the mycorrhizal associations of naturally established seedlings are the most informative for documenting the composition of the ectomycorrhizal fungal community after fires. The abundance of *Cistus* seedlings facilitates the stratification of sampling to address hypotheses regarding spatial distribution of inoculum as has been attempted in the present study. However, because of the nature of the soil it generally proved difficult to excavate seedlings to depths of more than 10 cm. Thus the activity of fungi in the deeper soil remains unknown. Although the immediate impact of fire is confined to the upper soil layer, sub-surface activity may be important to the recovery of some fungi. A particularly interesting question involves the depth to which mycorrhizal associations occur on plant roots in these ecosystems. Many plant species avoid drought-stress by locating roots deep inside fractures and crevices in the bedrock. Do mycorrhizal fungi play a role at these depths? The exposure of crevices by quarrying may provide access to deep roots to address this question.

The absence of E-Strain morphotypes from the greenhouse bioassay (Chapter 4) remains something of a mystery and does suggest the possibility that some aspect of the experimental conditions was inhibiting their development. It may be worthwhile repeating this experiment, paying particular attention to the watering regime, ensuring that soil moisture levels are not too high. The possibility also arises that other fungi were inhibited in forming ectomycorrhizas because of the experimental conditions. However, the morphotypes *Tuber* 1, *Inocybe* 1, *Genea*-like, Unknown 2 and Unknown 12 did form ectomycorrhizas, albeit infrequently, often capturing substantial proportions of root systems where present (see Figure 4.4). At present there is no way of telling whether these fungi were colonizing from mycorrhizal root fragments or from spores. Future experiments of this kind may best be targeted directly towards identifying spore populations by drying and sieving soils before planting them up.

## 7.9 Concluding remarks

In summing up I will address each of the questions that were posed as project aims at the beginning of this thesis.

Ectomycorrhizal fungal inoculum does appear to exhibit spatial patterning in the immediate aftermath of forest fires. This patterning appears to be related strongly to depth in the soil profile and weakly to proximity to resprouting shrubs. The weakness of the effect of microsite is most probably a reflection of little belowground structuring among roots of the resprouting shrubs. The relationship with soil depth may be explained by differences in post-fire colonization strategies and adaptations for fire survival of the fungi involved.

It appears that ectomycorrhizal fungi survive the impact of fires *via* one of two strategies. The majority of fungi appear to survive as mycelium beneath the top few centimetres of the soil, beyond the effects of lethal heating, recolonizing new roots as they become available. Other fungi survive the passage of fire as resistant propagules from which new mycelia develop and colonize new roots. Thus the dynamic of post-fire ectomycorrhizal fungal regeneration is analagous to that of the plants which has been described as an autosuccession in which the species that appear after the fire were all present in the forest in one form or another before the fire. Thus the mature forest fungi that regenerate from subterranean mycelia and those that regenerate from resistant propagules are the mycological counterparts of plant resprouters and seeders.

Compared to the unburned forest, most of the ectomycorrhizal fungi appear to be depressed by the action of fire. However, most of them appear to recover quickly and this is surely facilitated by the post-fire ubiquity of *Cistus* seedlings that help to ensure the continuity of these fungi.

By effectively heat sterilising the surface few centimetres of the soil, fire also creates a new niche that is exploited by certain fungi. These same fungi interact with *Cistus* seedlings from a very early stage in the plants development and may serve to provide the early access to external sources of nutrients that this small-seeded species requires. The fungi involved here benefit from a ready source of carbohydrate while at the same time increasing the chance of survival of the *Cistus* seedlings and thus their ability to facilitate the recovery of the mature forest fungi.

Thus, like the aboveground vegetation, ectomycorrhizal fungal communities appear to be resilient to the effects of fire in Mediterranean pine forests through a finely balanced system of interaction and feedback. However, this may apply only to situations in which fire return times lie within the range of adaptation of the vegetation. Where fire return times are shorter than this adaptive range problems may arise. Where fires re-occur at the same site before seeders have had a chance to produce sufficient seed stores to promote full regeneration a second time, the floristic composition of that site will change. In particular, there will be a reduced flush of *Cistus* seedlings for ectomycorrhizal fungi to colonize. Under such circumstances continuity of ectomycorrhizal fungi may be more dependent on the refugial effects of resprouting shrubs. It is also in these circumstances that intervention is required if tree cover is to be restored on these sites. Afforestation programmes may then be able to exploit the refugial effect of resprouting shrubs by planting close to them to optimize colonization by ectomycorrhizal fungi. Trials need to be carried out to assess the benefits that might arise from such a planting strategy.

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# Appendix 1. Form used for description of morphotypes

# Appendix 2. Descriptions of ectomycorrhizas of *Cistus creticus* L.

The descriptions that follow are arranged by morphotype name in alphabetical order.

In the descriptions, where a colour is followed by a number in parentheses, e.g. Buff (52), this refers to colours listed in the Colour Identification Chart for the Flora of British Fungi (Royal-Botanic-Garden-Edinburgh, 1969). Colours without numbers in parentheses are descriptive terms attributed to the present author.

The principle source for protocols and terminology used in the description of ectomycorrhizas was the series of volumes entitled "Descriptions of Ectomycorrhizae" (Agerer *et al.*, 1996-2001) and the accompanying set of photographic plates, "Colour Atlas of Ectomycorrhizae" (Agerer, 1987-2001; Agerer *et al.*, 1996-2001). In the present descriptions where characters are attributed in parentheses to a Type, e.g. outer mantle (Type B), these refer to the character types described at the beginning of "Descriptions of Ectomycorrhizae" (Agerer *et al.*, 1996-2001). Additional sources of information have been derived from two other sources: "Identification of Ectomycorrhizas" (Ingleby *et al.*, 1990) and "A Manual of Concise Descriptions of North American Ectomycorrhizae" (Goodman *et al.*, 1996-2000).

Where captions to photographs in the present descriptions include the term 'Cotton blue', this relates that the photograph is of material mounted in Cotton blue under a cover slip. Where the term 'DIC' appears, this relates that the photograph is taken using Differential Interference Contrast.

## A2.1 Ascomycete 1 (JMCc17)

### Macroscopic features

***Morphology*** – Simple unbranched or simple monopodial often arising directly from the primary root (Figure A2.1a, b). Swollen, especially at tips. Tips straight to bent. 1-1.5 x 0.4 mm. Faint reticulate pattern sometimes visible on surface (due to the large cells of the mantle being partially visible). Tips are usually smooth, rarely slightly cottony. Colour can be quite variable, ranging between specimens from Buff (52) to Saffron (49).

### Microscopic features

***Hyphae*** – 4 - 6 µm diameter. Straight to tortuous, occasionally curved. Frequently elbowed. Usually smooth, rarely finely and sparsely verrucose/granular. Branching common. Septa common, not clamped. Blunt tipped.

***Mantle*** – 25 - 28 µm thick. Large mantle cells obvious in section (Figure A2.1e). In common with ectomycorrhizas formed by *Cistus creticus* with other fungi, the Hartig net extends into the second layer of root cells (Figure A2.1e).

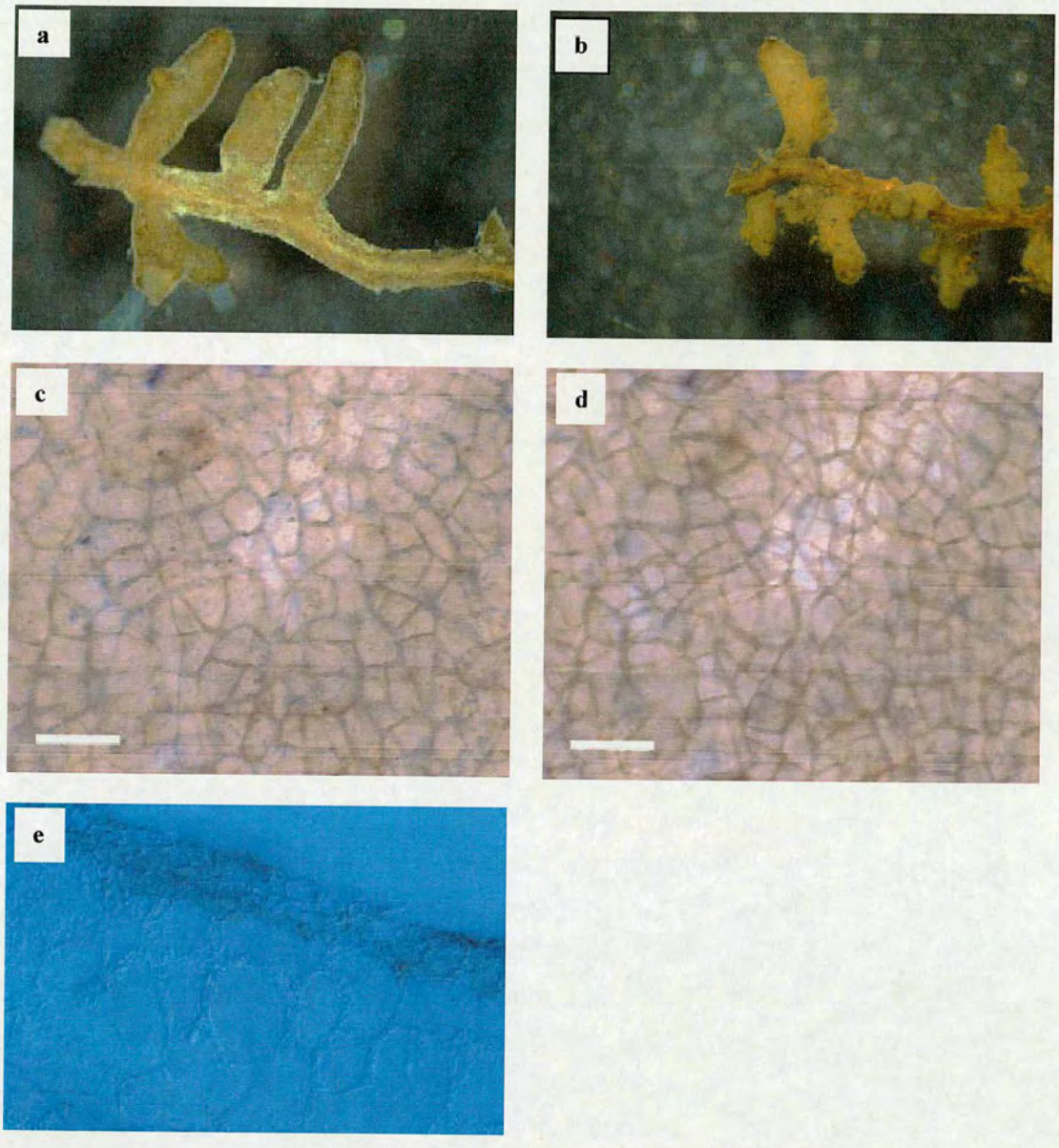
***Outer mantle*** – Pseudoparenchyma with large, irregular sized, rounded to angular cells (FigureA2.1c).

***Inner mantle*** – Pseudoparenchyma as in outer mantle but with cells becoming more angular (Figure A2.1d).

### Identity

Ascomycete of unknown identity. Identified as an ascomycete by the presence of Woronin bodies associated with the septa. Similar to ITE 4 (Ingleby *et al.* 1990).

Figure A2.1. Ascomycete 1.
(a), (b) Simple and monopodially branched root tips arising from the primary root. (c) Outer mantle (bar = 25 µm). (d) Inner mantle (bar = 25 µm). (e) Longitudinal section showing mantle and Hartig net (DIC).



## A2.2 Ascomycete 2 (JMCc55)

### Macroscopic features

***Morphology*** – Simple unbranched to monopodial pinnate. Pale cream colour when young (Figure A2.2a ), darkening to a light brown colour with age (Figure A2.2b). Tips straight to bent. Cottony, especially when older.

### Microscopic features

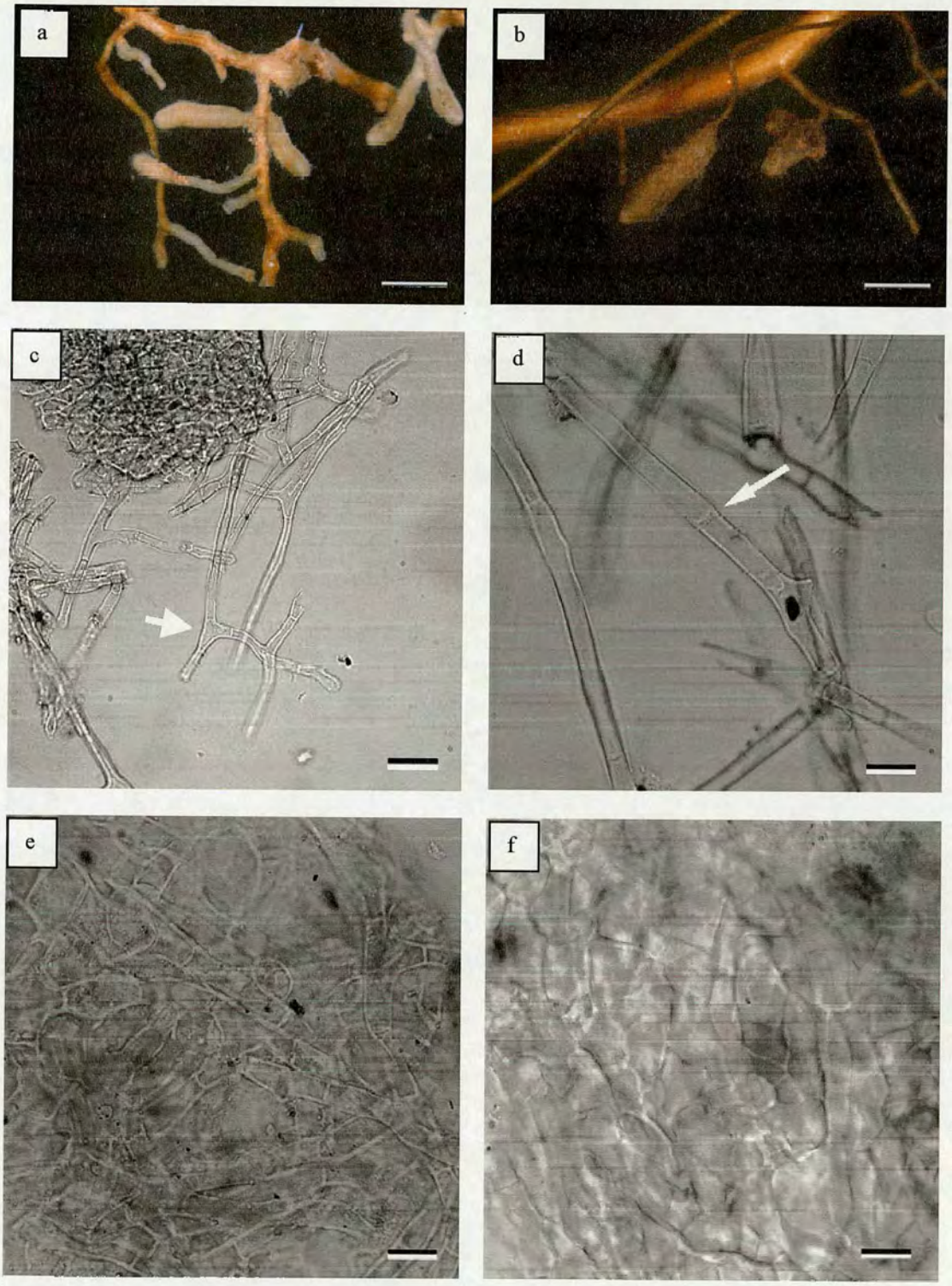
***Hyphae*** – Emanating hyphae abundant. 4 – 5 μm diameter. Hyaline. Straight to curved to tortuous. Smooth and thick-walled. Contact and H-shaped anastomoses frequent. Branching common. Branching intersections often enlarged with three hyphae sub-tending at $120^0$ angles. Branching also squarrose or 'Y-shaped' (Figure A2.2c). Woronin bodies spherical (Figure A2.2d).

***Mantle*** – Outer mantle a plectenchyma of branched septate hyphae (Figure A2.2e). Middle and inner mantle layers a pseudoparenchyma of irregular rounded to angular cells (Figure A2.2f). Mantle cells become smaller towards the root.

### Identity

The presence of Woronin bodies associated with the septa indicate that this is a species of Ascomycete.

Figure A2.2 Ascomycete 2.
(a) Young mycorrhizas (bar = 1mm). (b) Old mycorrhizas (bar = 1 mm). (c) Branched hyphae. Arrow indicates enlarged intersection (bar = 25 μm). (d) Hypha with Woronin bodies at septum (bar = 10 μm). (e) Outer mantle (bar = 10 μm). (f) Inner mantle (bar = 10 μm).



# A2.3 Bankeroid 1 (JMCc36)

## Macroscopic features

***Morphology*** – Simple to monopodial pinnate. Colour Sepia (26) to Drab (33). Tips cottony.

***Rhizomorphs*** – Rare. Round in cross-section.

## Microscopic features

***Rhizomorphs*** – Compact but undifferentiated (Figure 2.3a). Margins somewhat 'hairy' with loose hyphae. Rhizomorph hyphae are morphologically similar to the emanating hyphae.

***Hyphae*** – 2-4 µm. Uniform. Common to abundant. Septate. Branched (branching often squarrose) (Figure 2.3b). Occasional anastomoses (Figure 2.3c).

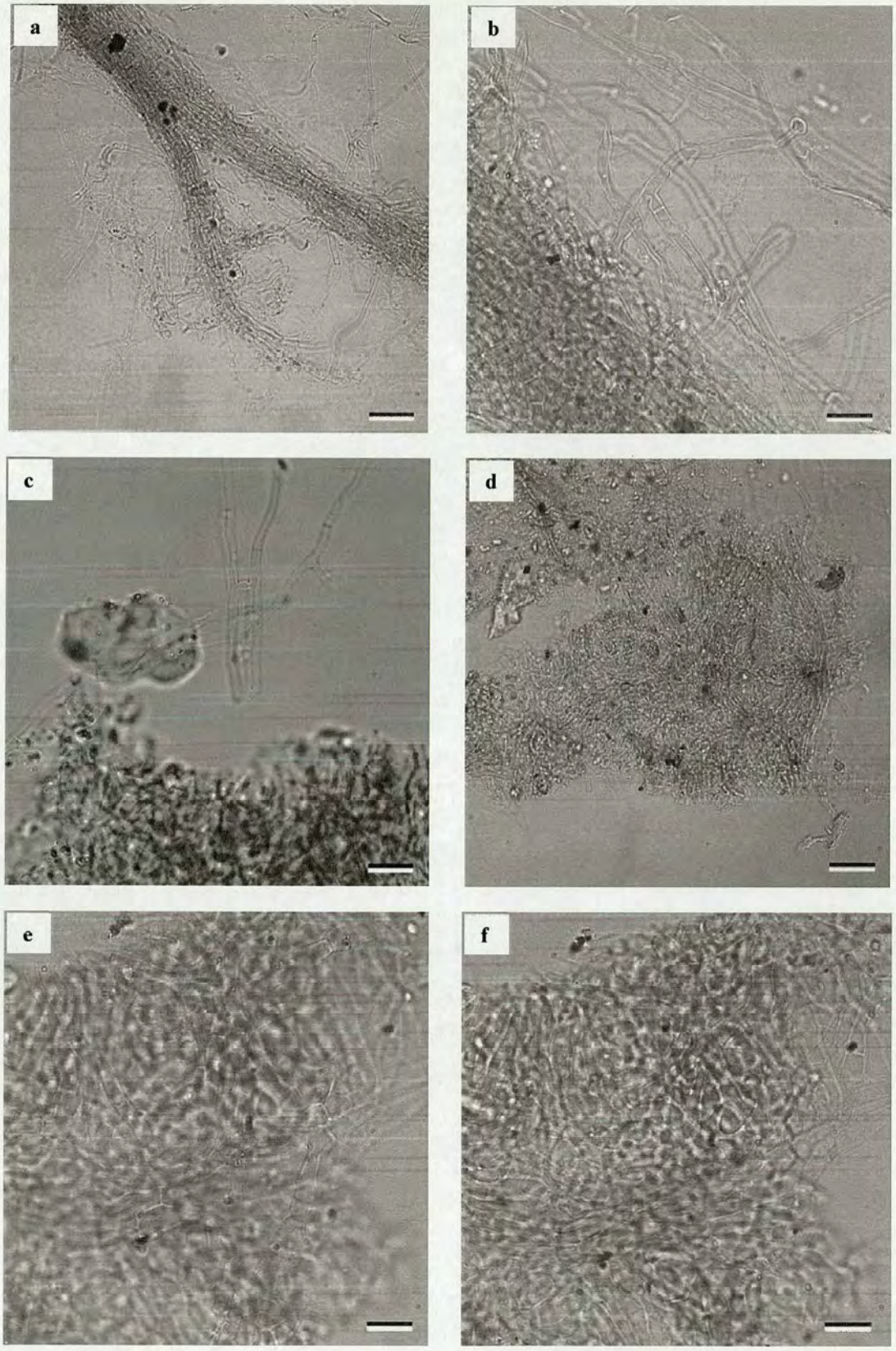
***Mantle*** – Plectenchymatous throughout. Hyphae irregularly arranged with no particular pattern discernible (Agerer Type B) (Figure 2.3d, e, f).

## Identity

Closely resembles morphotypes associated with species of *Phellodon, Bankera* and *Hydnellum* in having a plectenchymatous mantle of fairly uniform hyphae without clamp connections and compact but undifferentiated rhizomorphs (Agerer & Otto, 1997).

Figure A2.3 Bankeroid 1 (next page). (a) Rhizomorph compact, undifferentiated with hairy margins (bar = 25 µm). (b) Emanating hyphae uniform, squarrosely branched (bar = 10 µm). (c) Hyphal anastomosis (bar = 10 µm). (d) Mantle dissection showing hyphae arranged as an irregular plectenchyma (bar = 25 µm). (e) and (f) Mantle dissection showing hyphae arranged as an irregular plectenchyma (bar = 10 µm).

Figure A2.3 Bankeroid 1.
(caption on previous page)



# A2.4 Basidiomycete 1 (JMCc29)

## Macroscopic features

***Morphology*** – Simple, unbranched or irregular branching (Figure A2.4a, Figure A2.4b). Tips straight or bent. Cottony with abundant emanating hyphae forming 'cloud' around mycorrhiza. Often felty, frequently with hyphae emanating from the distal ends (Figure A2.4a). Matte.

***Rhizomorphs*** – Rare. Smooth. Round in cross-section. Unbranched.

## Microscopic features

***Rhizomorphs*** – Very simple, undifferentiated (Agerer Type B). Formed from a small number of hyphae only slightly interwoven (Figure A2.4c).

***Hyphae*** – 2-4 µm diam. Straight to curved, frequently tortuous. Smooth. Branching common, often squarrose (Figure A2.4d). Septa common, clamped. No colour reaction in cotton blue. Hyphae remain lightly tinted brown.

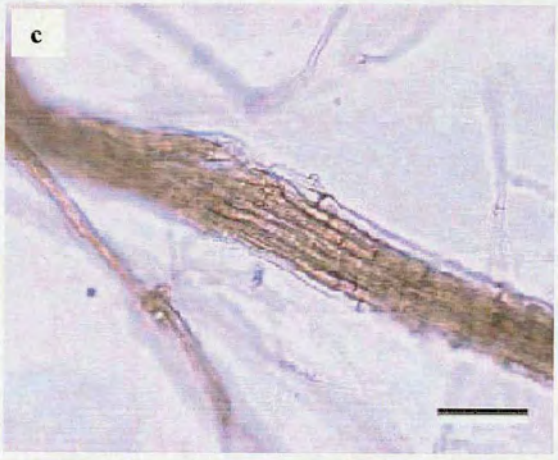
***Mantle*** – plectenchymatous throughout (Agerer Type B). Overlain in places by longitudinally running bundles of clamped hyphae (bundles 4 - 8 µm wide) (Figure A2.4e).

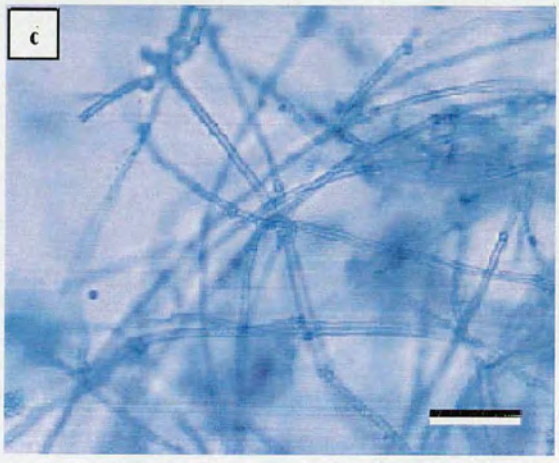
## Identity

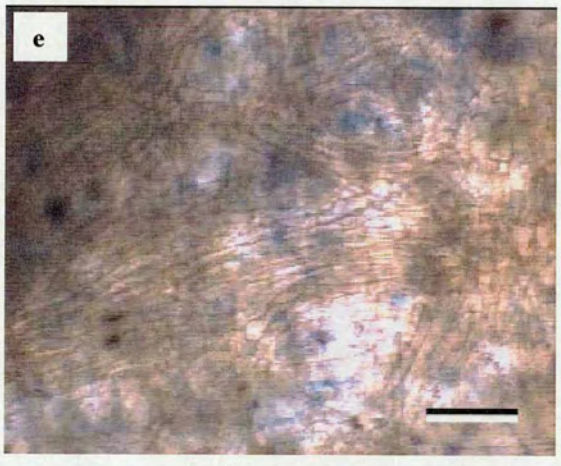
Presence of clamp connections identify this fungus as a basidiomycete.

Figure A2.4. Basidiomycete 1.
(a) Monopodial pinate branching (bar = 2 mm). (b) Unbranched mycorrhiza (bar = 2 mm). (c) Simple, undifferentiated rhizomorph (bar = 25 µm). (d) Emanating hyphae clamped and squarrosely branched (Cotton blue, bar = 25 µm). (e) Bunches of hyphae on surface of plectenchymatous mantle (bar = 25 µm).





## A2.5 Basidiomycete 2 (JMCc47)

### Macroscopic features

*Morphology* – Simple, unbranched, straight tips. Brownish in colour. Sparsely covered with short emanating hyphae.

### Microscopic features

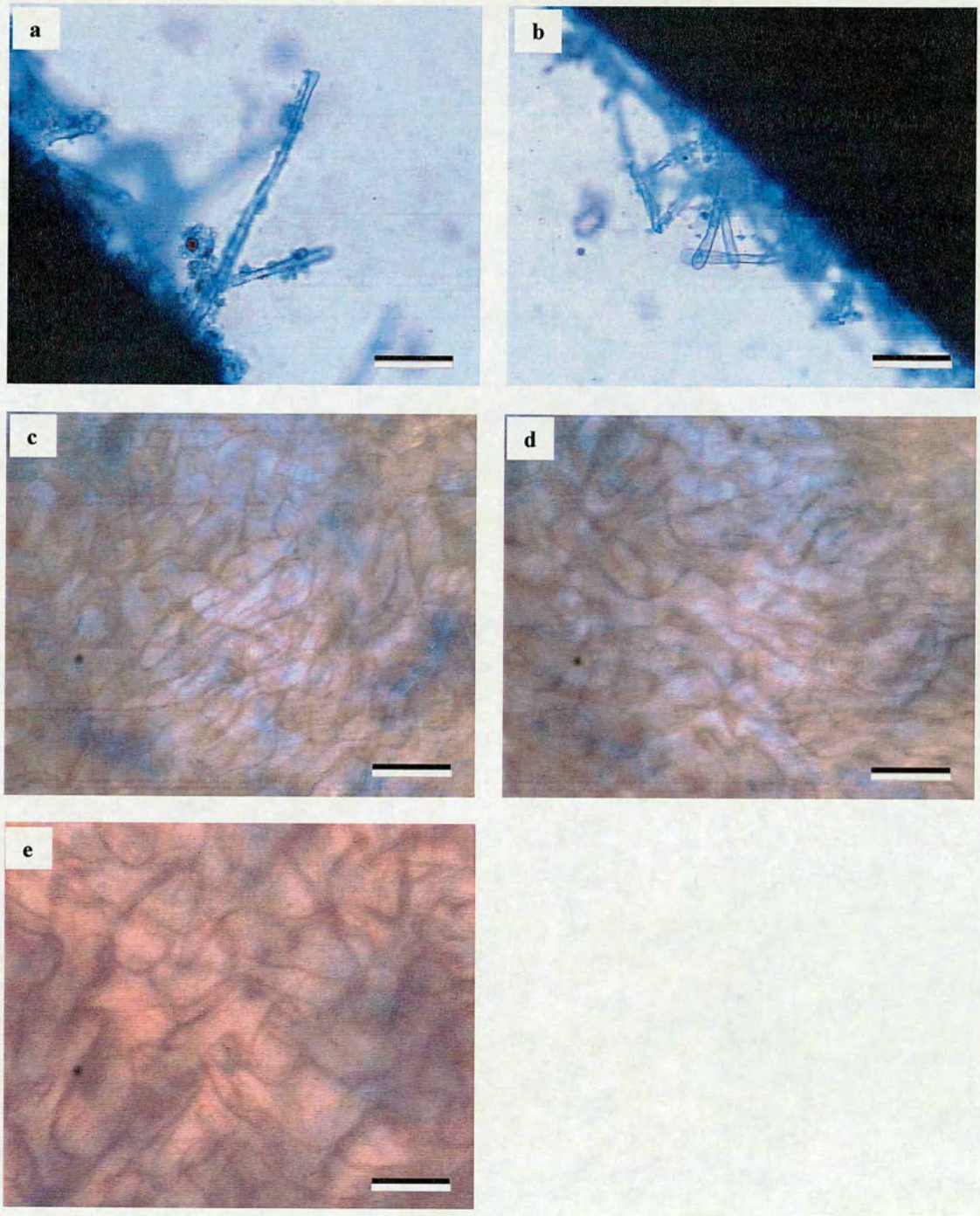
*Hyphae* – Common, 3 - 4 µm diameter. Very short (up to 100 µm). More or less straight, occasionally branched. Tips often slightly swollen (Figure A2.5a, A2.5b). Septa common and clamped.

*Mantle* – Densely plectenchymatous with no obvious pattern in the outer (Figure A2.5c) and inner (Figure A2.5d) mantle. Hyphal cells in mantle typically 2 - 3 µm diameter. Appear somewhat thicker towards the distal end (Figure A2.5e).

### Identity

Basidiomycete of unknown identity

Figure A2.5 Basidiomycete 2.
(a), (b) Emanating hyphae (bar = 25 μm). (c) Outer mantle (bar = 10 μm). (d) Inner mantle (bar = 10 μm). (e) Outer mantle at distal end of root tip (bar = 10 μm)



# A2.6 Basidiomycete 3 (JMCc50)

## Macroscopic features

***Morphology*** – Clusters of irregularly branched root tips (Figure A2.6a). Cottony texture. Usually densely covered with soil particles. Tips straight to bent.

***Rhizomorphs*** - Common to occasional. Up to 80 μm diameter. Tan to yellowish colour.

## Microscopic features

***Rhizomorphs*** - Slightly hairy in appearance due to hyphae breaking away from the main body of the rhizomorph (Figure A2.6b). Undifferentiated.

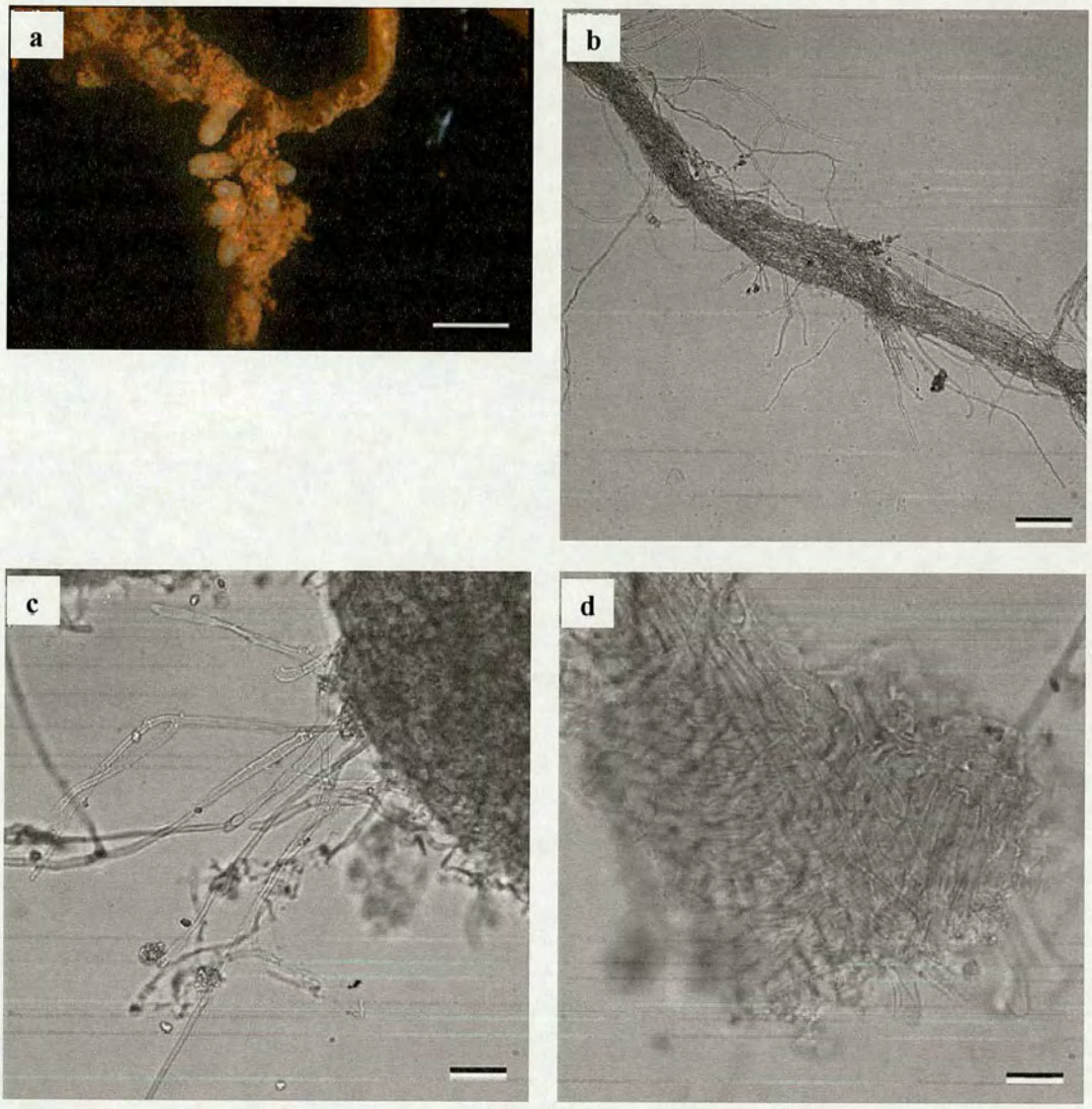
***Hyphae*** – Usually abundant. 2 – 2.5 μm diameter. Curved to occasionally tortuous. Clamped septa common. Hyphae hyaline and smooth-walled. Squarrose to 'Y-shaped' branching common. (Figure A2.6c)

***Mantle*** – Plectenchymatous throughout. Mantle hyphae branched, interlocking (Figure A2.6d). Occasionally lying in uni-directional swathes in the outer mantle layers.

## Identity

Unknown Basidiomycete.

Figure A2.6 Basidiomycete 3.
(a) Cluster of mycorrhizal root tips (bar = 1 mm). (b) Rhizomorph (bar = 25 μm). (c) Emanating hyphae (bar = 25 μm). (d) Outer mantle (mantle dissection, bar = 10 μm).



# A2.7 Basidiomycete 4 (JMCc57)

## Macroscopic features

***Morphology*** – Clusters of irregularly branched root tips. Densely cottony at base of clusters with many soil particles adhering.

***Rhizomorphs*** – Frequent. White.

## Microscopic features

***Rhizomorphs*** – Compact but with hairy margins due to emanating hyphae (Figure A2.7a). Undifferentiated (Figure A2.7b).

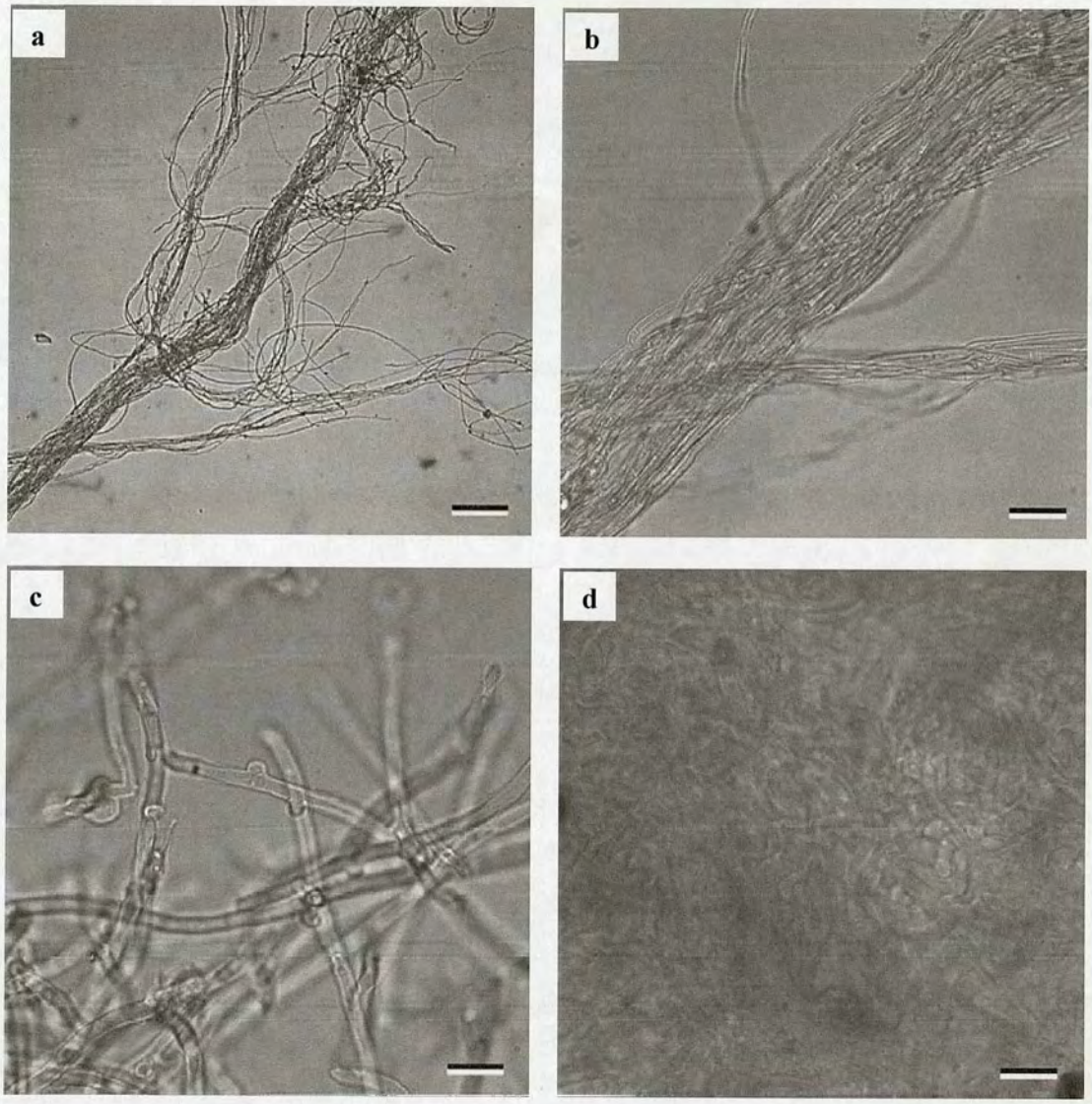
***Hyphae*** – Emanating hyphae abundant. 2 – 3 μm diameter. Curved or wavy to tortuous. Hyaline. Smooth to finely verrucose. Clamped septa common (Figure A2.7c). Squarrose branching common.

***Mantle*** – Plectenchymatous throughout (Agerer Type B) (Figure A2.7d)

## Identity

Unknown Basidiomycete

Figure A2.7 Basidiomycete 4.
(a) Rhizomorph with hairy margins (bar = 100 μm). (b) Rhizomorph undifferentiated (bar = 25 μm). (c) Hyphae with clamps (bar = 10 μm). (d) Outer mantle (bar = 10 μm).



# A2.8 Basidiomycete 5 (JMCc33)

## Macroscopic features

***Morphology*** – Tips 2 x 0.5 mm. Simple (Figure A2.8a) or irregularly branched. Very pale cream colour, almost white. Tips straight, smooth, matte (Figure A2.8b). Mantle fully developed.

## Microscopic features

***Hyphae*** – Rare. Two types:
1. Thin (2 - 3 μm), tortuous, unclamped. Occasionally aggregated to form a primitive-rhizomorph emanating from the mantle surface.
2. Thicker (4 - 5 μm), straight, clamped, occasionally branched (Figure A2.8c, Figure A2.8d)

***Mantle*** –

***Outer mantle*** – Usually plectenchymatous (Figure A2.8e) but with frequent patches that appear pseudoparenchymatous. Hyphal diameter small (< 2 μm). Some cells are epidermoid, some are irregular and angular.

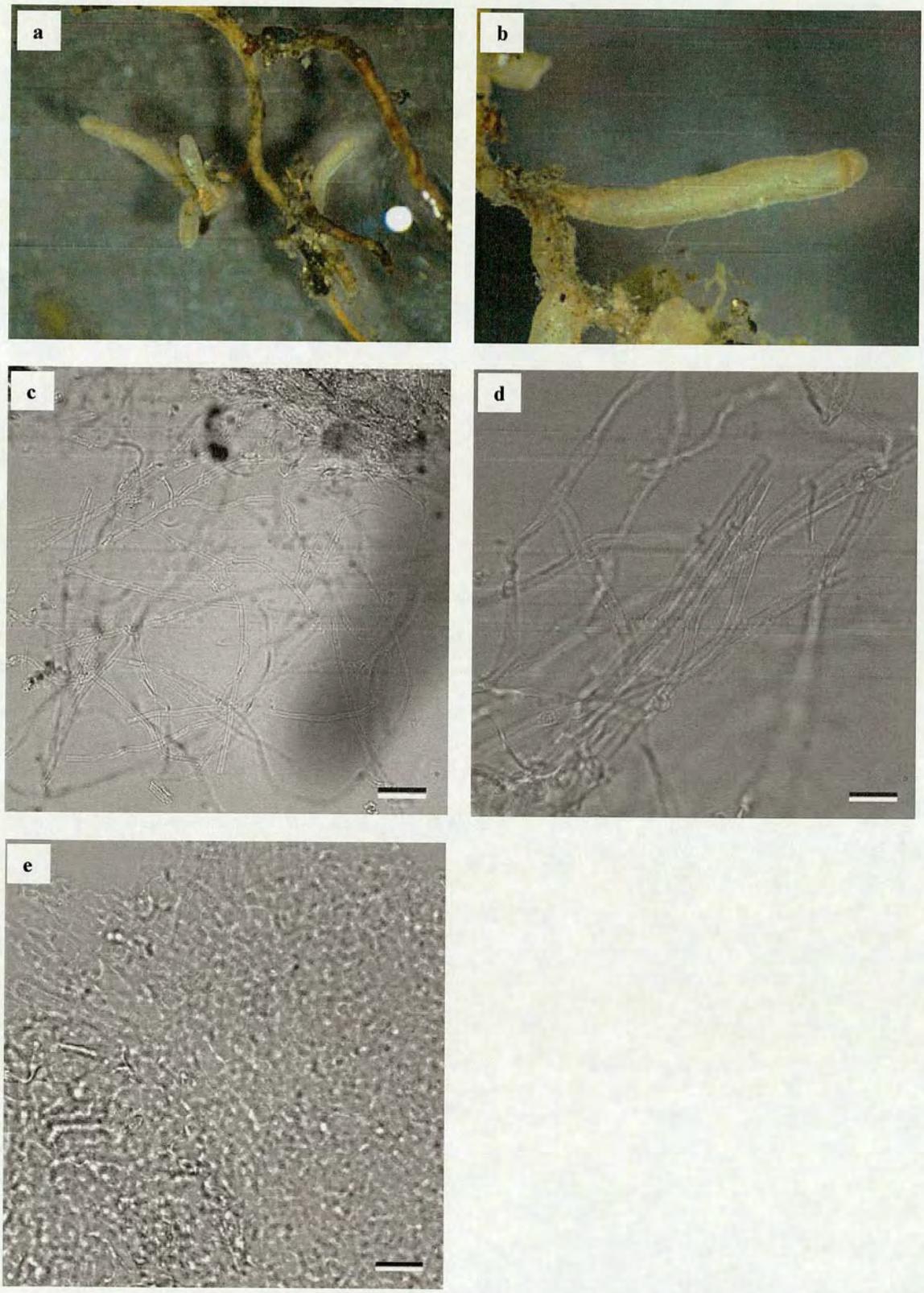
***Inner mantle*** – Entirely plectenchymatous. Cells very fine.

## Identity

Unknown basidiomycete.

Figure A2.8 Basidiomycete 5 (next page). (a) Tips simple, unbranched. (b) Tips pale, straight, smooth. (c)Type 2 hyphae. Scale = 25 μm. (d) Type 2 hyphae, clamp connections. Scale = 10 μm. (e) Plectenchymatous outer mantle. Scale = 10 μm.

Figure A2.8 Basidiomycete 5
(caption on previous page)



# A2.9 *Cenococcum geophilum* (JMCc11)

## Macroscopic features

***Morphology*** – This distinctive morphotype is characterised by its black, granular mantle and long dark emanating hyphae (Figure A2.9a, Figure A2.9b). Root tips are generally small (1 -2 mm long) and unbranched.

## Microscopic features

***Hyphae*** – 4 - 6 µm diameter, straight, distinctly septate. Brown in colour.

***Mantle*** –

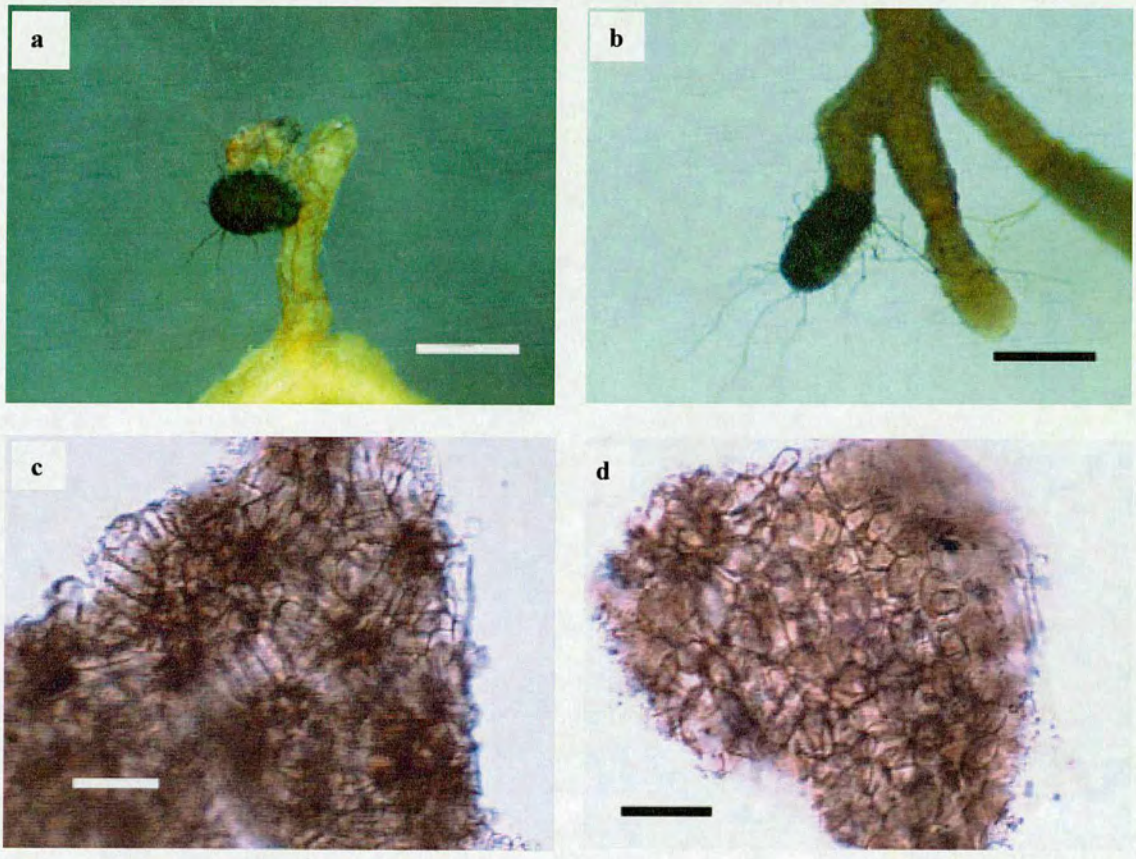
***Outer mantle*** – Distinctive clusters of elongated cells emanating radially from a central, isodiametric cell or group of cells, forming characteristic star-shaped patterns (Figure A2.9c). The fungal cells are generally thick-walled.

***Inner mantle*** – Pseudoparenchyma composed of more or less isodiametric, rounded cells without thickened walls (Figure A2.9d).

## Identity

*Cenococcum geophilum* is one of the most widely reported ectomycorrhizal symbionts and is easily identified by its black granular mantle and distinctive dark, thick emanating hyphae.

Figure A2.9 Cenococcum geophilum.
(a) and (b) Simple root tips with typical dark emanating hyphae (bars = 2 mm). (c) Mantle dissection showing radiating clusters of cells in the outer mantle layer (bar = 25 µm). (d) Mantle dissection showing pseudoparenchyma of inner mantle layer (bar = 25 µm).



# A2.10 E-Strain (JMCc31)

## Macroscopic features

*Morphology* – Tips simple, unbranched, bent to tortuous (Figure A2.10a, Figure A2.10b). Occasionally simply branched. Distal ends sometimes swollen when young (Figure A2.10c). Light to chestnut brown in colour. Distal ends often appear paler where the mantle does not enclose the entire root. Few emanating hyphae. Mantle surface more or less smooth.

## Microscopic features

*Hyphae* – (4-) 7.5 - 8 (-12) µm diameter. Light brown in colour. Thick-walled. Smooth to finely verrucose (Figure A2.10d). More likely to be finely verrucose away from the mantle edge. Branching common. Septa simple, with Woronin bodies (Figure A2.10e) and occasional septal plugs (Figure A2.10f).

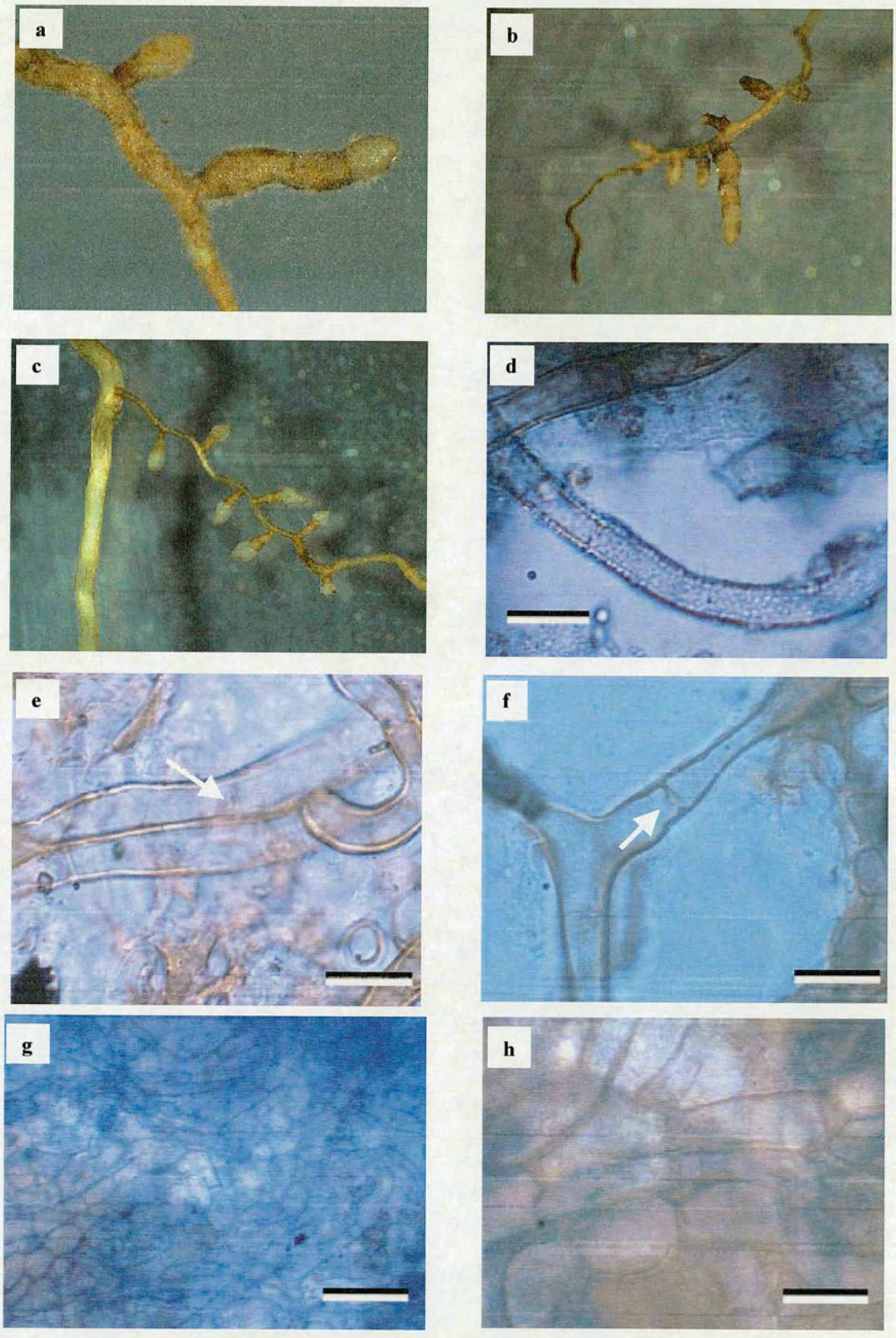
*Mantle* – Weakly developed. Thin (1-2 fungal cells). Discontinuous network of swollen hyphae (Figure A2.10g). Hyphae may appear constricted at septa (Figure A2.10h).

## Identity

The hyphal and mantle characteristics strongly point towards this fungus belonging to the E-strain aggregate. Longitudinal sectioning revealed that intracellular haustoria were absent from this 'E-strain' morphotype and thus it may be classified as a weak ectomycorrhiza.

Figure A2.10 E-Strain (next page). (a) Tips bent to tortuous. (b) Colonized tips often occur as clusters arising from the primary root. (c) Young colonized root tips appear swollen at their distal ends. (d) Hyphae often verrucose, especially away from the mantle surface (Cotton blue, bar = 10 µm). (e) Woronin bodies (indicated by arrow) (bar = 10 µm). (f) Septal plug (indicated by arrow) (DIC, bar = 10 µm). (g) Mantle a thin, discontinous network of swollen hyphae (bar = 25 µm). (h) Mantle hyphae swollen and constricted at the septa (bar = 10 µm).

Figure A2.10 E-Strain
(caption on previous page).



# A2.11 *Genea*-like (JMCc51)

## Macroscopic features

***Morphology*** – Generally simple, unbranched, occasionally with simple monopodial branching (Figure A2.11a). Cigar brown (16) to Fuscous black (36). Root tips slightly to very cottony with brown emanating hyphae (Figure A2.11b).

## Microscopic features

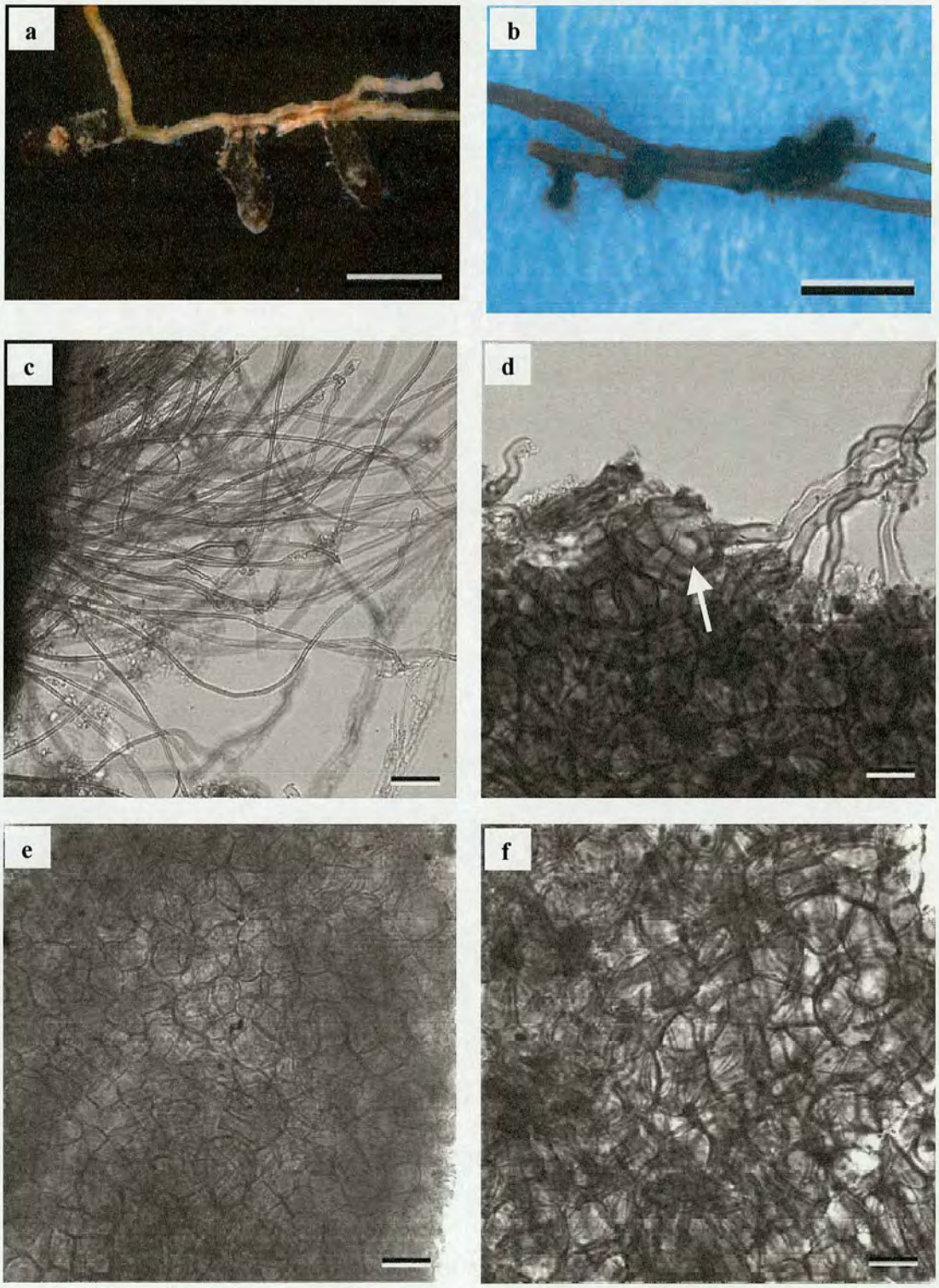
***Hyphae*** – Rare to common, occasionally abundant. 2 – 4 μm diameter. Straight to curved to tortuous. Thick-walled, light brown, unbranched. Smooth-walled and regular close to mantle surface. Often becoming inflated and irregular at distal ends (Figure A2.11c). Often seen emanating from large, swollen basal cells embedded in the outer mantle (Figure A2.11d).

***Mantle*** – Pseudoparenchymatous with rounded to angular cells in the outer layer (Figure A2.11e, A2.11f). Outer mantle cells 6 – 12 μm diameter, rather irregular. Mantle cells becoming more rounded in middle layers and finally becoming plectenchymatous in the inner layer.

## Identity

The tip morphology, light brown, thick-walled emanating hyphae with inflated tips and the angular cells of the pseudoparenchymatous outer mantle are very similar to descriptions of *Genea verrucosa* Vitt. + *Quercus* sp. (Jakucs *et al.*, 1998) and *Genea hispidula* Berk. et Br. + *Fagus sylvatica* (Brand, 1991).

Figure A2.11 *Genea*-like.
(a) Simple tips arising from lateral root (bar = 1 mm). (b) Mycorrhizas with abundant emanating hyphae (bar = 1 mm). (c) Emanating hyphae (bar = 25 μm). (d) Swollen basal cell of emanating hypha (arrow, bar = 10 μm). (e) Outer mantle with rounded cells (bar = 25 μm). (f) Outer mantle with rounded to angular cells (bar = 10 μm).



# A2.12 *Inocybe* 1 (JMCc46)

## Macroscopic features

***Morphology*** – Simple, straight tips. Smooth to cottony. Mantle well developed (host not visible). Pale cream colour.

***Rhizomorphs*** – Occasional.

## Microscopic features

***Rhizomorphs*** – Simple, undifferentiated. Formed from clamped hyphae (Agerer Type 1) (Figure A2.12a).

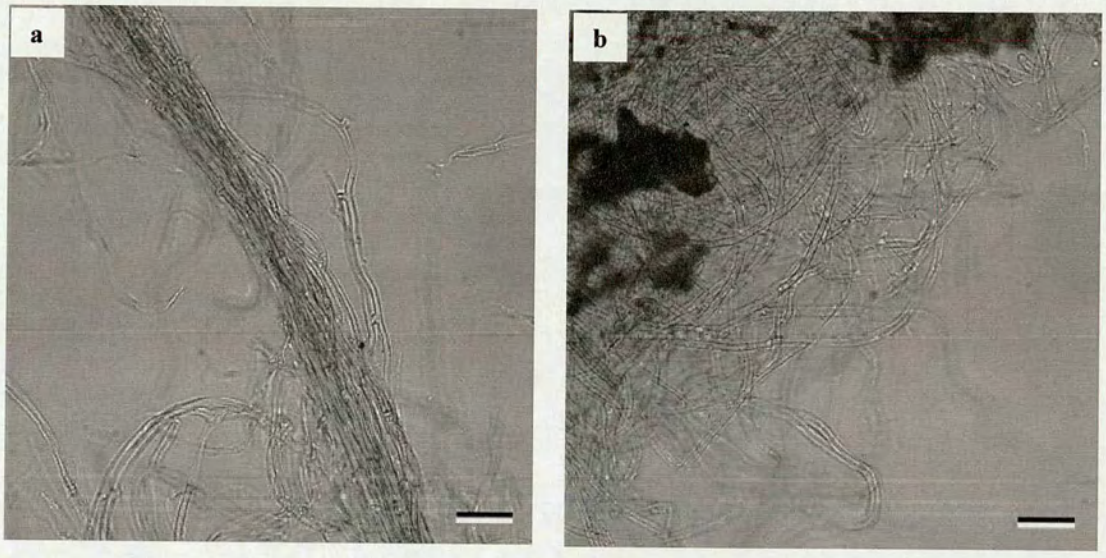
***Hyphae*** – 2 types
1. (Figure A2.12b) Common to rare. 2 - 4 µm diameter. Curved to tortuous. Hyphal cell walls smooth and even. Septa rare and clamped. Clamp connections large (> 2/3 hyphal diameter). Hyphae unbranched. Hyphal cells filled with oil droplets. Stain dark blue in Cotton blue.
2. Rare (but occasionally dominant). Thinner (2 µm max.). More even-walled. Septa rare, indistinct, unclamped.

***Mantle*** – Irregular plectenchyma (Agerer Type B).

## Identity

Identified as *Inocybe* by the distinctive hyphae that have proportionately large clamp connections (often equal in diameter to the hyphae) and plectenchymatous mantle. The lack of a gelatinous matrix in the mantle suggests that the fungus probable falls within *Inocybe* subg. *inocybe* (Beenken *et al.*, 1996).

Figure A2.12 *Inocybe* 1.
(a) Rhizomorph (bar = 25 μm). (b) Emanating hyphae (bar = 25 μm).



# A2.13 *Inocybe* 2 (JMCc34)

## Macroscopic features

***Morphology*** – Tips simple or monopodial pinnate. When pinnate, branching rare. Tips straight or occasionally bent. Densely cottony with abundant emanating hyphae (Figure A2.13a).

***Rhizomorphs*** – Rare, slightly 'hairy', round in cross-section, unbranched.

## Microscopic features

***Rhizomorphs*** – Undifferentiated, primitive, consisting of a few loosely woven hyphae (Figure A2.13b).

***Hyphae*** – Abundant, 2-4 µm diameter, tortuous. Septa common and clamped. Clamps tend to be large ( usually equal to the diameter of the hypha). Clamps occasionally with aperture (Figure A2.13c). Hyphae smooth and commonly branched. Branching usually squarrose. Occasional H-shaped anastomoses with clamp on bridging hypha (Figure A2.13d). Oil-like inclusions common. Hyphae stain blue in Cotton Blue, purple to pale blue in Toluidine Blue.

***Mantle*** –

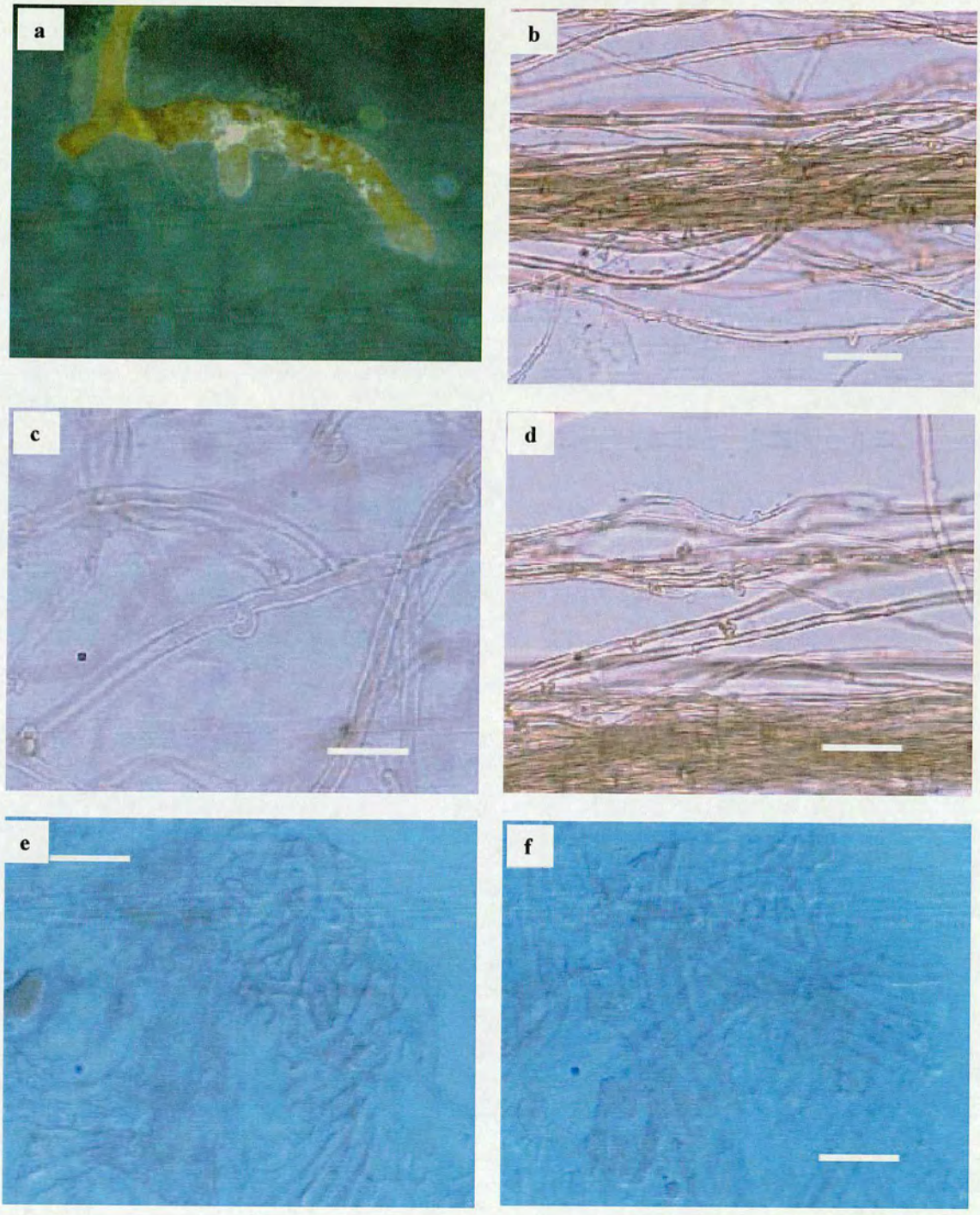
***Outer mantle*** – Plectenchyma embedded in gelatinous matrix (Figure A2.13e).

***Inner mantle*** – Plectenchyma embedded in gelatinous matrix (Figure A2.13f).

## Identity

Identified as *Inocybe* by the distinctive hyphae that have proportionately large clamp connections (often equal in diameter to the hyphae) and plectenchymatous mantle. The presence of a gelatinous matrix in the mantle of this morphotype probably places it within *Inocybe* subg. *mallocybe* (Beenken *et al.*, 1996).

Figure A2.13. *Inocybe* 2.
(a) Emanating hyphae abundant. (b) Rhizomorph primitive (bar = 25 µm). (c) Clamp connection with central aperture (bar = 10 µm). (d) Anastomosis with clamp connection on bridging hypha (bar = 25 µm). (e) Outer mantle (DIC, bar = 10 µm). (f) Inner mantle (DIC, bar = 10 µm).



## A2.14 *Thelephora terrestris* (JMCc49)

### Macroscopic features

***Morphology*** – Monopodial pinnate to irregularly branched (Figure A2.14a). Individual tips straight to slightly bent. Colour fulvous (12) with distal ends generally paler, greyish (Figure A2.14b). Surface texture felty, commonly with short spines.

### Microscopic features

***Hyphae*** – Emanating hyphae abundant. 3 – 4 μm diameter. Curved to wavy. Hyaline. Septa common, both clamped and unclamped. Hyphal branching occasional. Clamped septa often observed at branch intersections (Figure A2.14c).

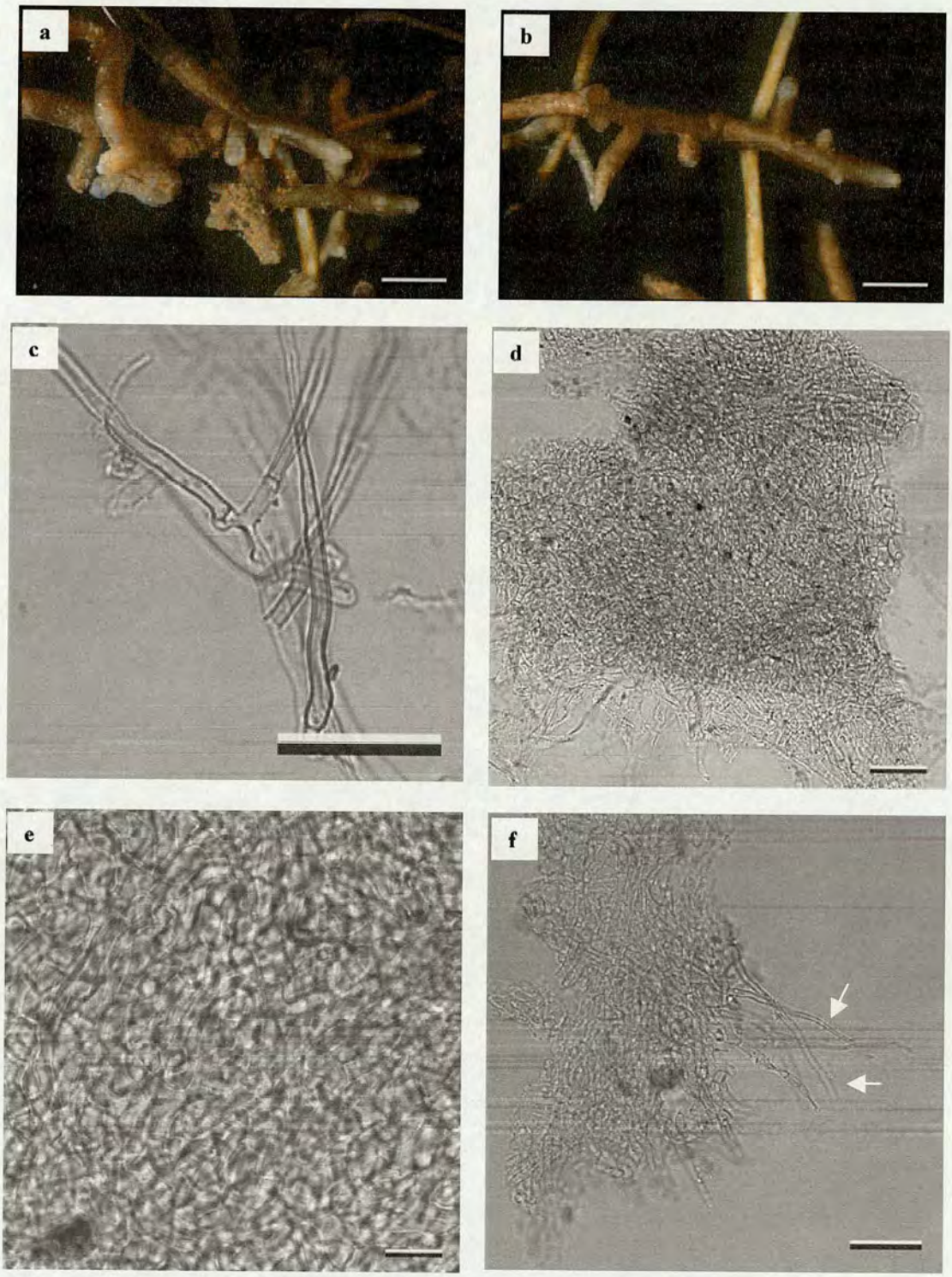
***Mantle*** – Plectenchymatous throughout (Figure A2.14d). Mantle hyphae broad (4 – 6 μm) and branched forming densely intertwining layers (Figure A2.14e).

***Cystidia*** – Awl-shaped, curved to wavy (Figure A2.14f). 3 μm at base narrowing to 1 μm at the tips. Up to 80 μm long. Often with basal clamp.

### Identity

This morphotype was confirmed as *T. terrestris* by comparison with EM roots growing under fruiting bodies in pot-grown *Cistus creticus* plants.

Figure A2.14 Thelephora terrestris.
(a) Clusters of simply branched mycorrhizas (bar = 1 mm). (b) Tips of mycorrhizas pale (bar = 1 mm). (c) Clamp connections at hyphal branch (bar = 25 μm). (d) Mantle dissection showing hyphae arranged as irregular plectenchyma (bar = 25 μm). (e) Close-up of outer mantle (bar = 10 μm). (f) Mantle dissection showing cystidia (arrows, bar = 25 μm).



# A2.15 Thelephoroid 1 (JMCc39)

## Macroscopic features

***Morphology*** – Simple unbranched or simply branched tips (Figure A2.15a). Tips straight. Appear woolly with thick mass of brown setae on mantle surface (Figure A2.15b). Colour Bay (19) to Cigar Brown (16).

## Microscopic features

***Hyphae*** – Two types:
1. Long hyphae (Figure A2.15c) – Occasional. 4 µm diameter, septa rare and obscure, oil bodies common, squarrose branching common, no stain reaction in Cotton Blue (hyphae remain light brown).
2. Short hyphae – Rare. 5-6 µm diameter, septa common and obvious, stain blue in Cotton Blue.

***Mantle*** –

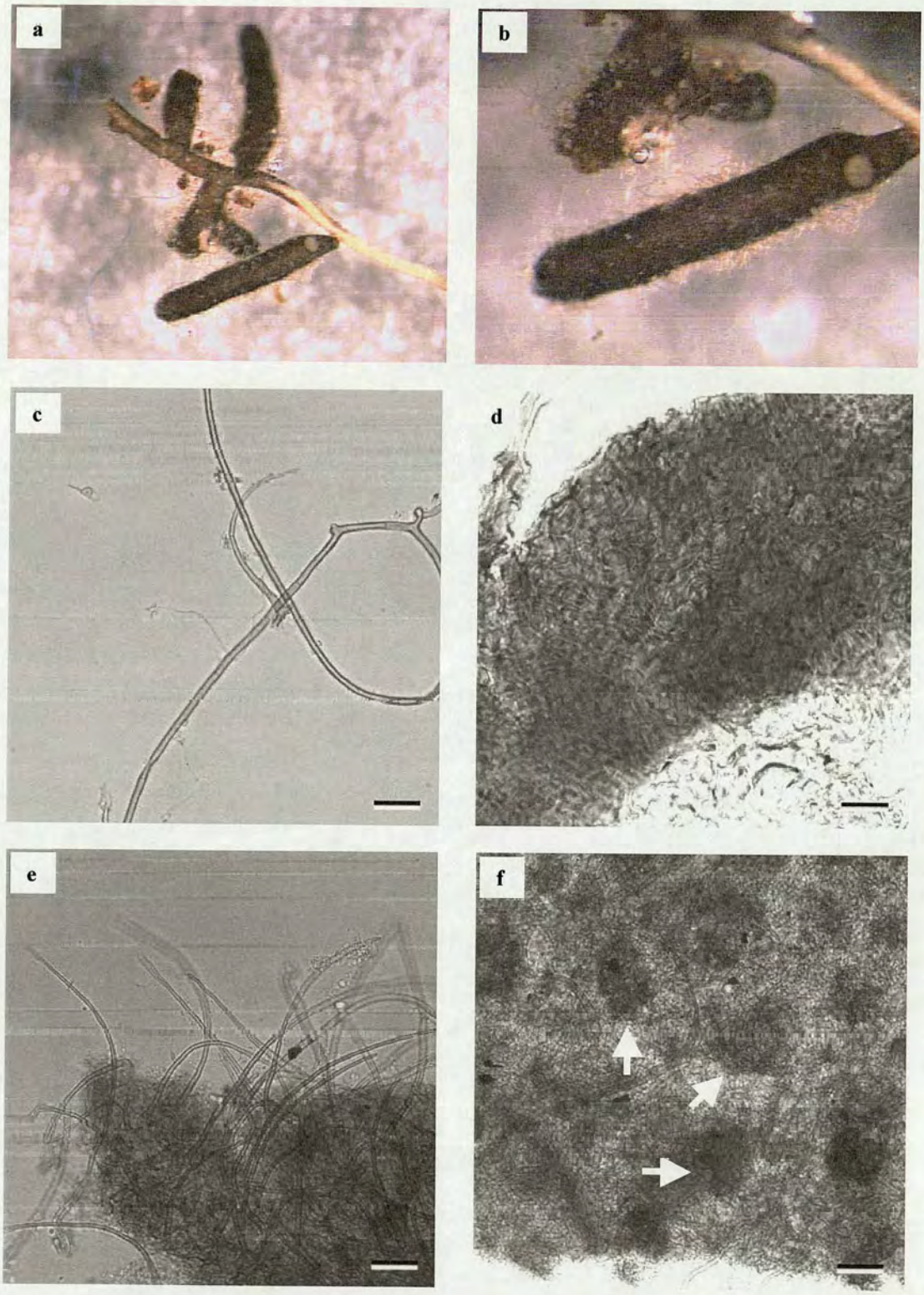
***Outer mantle*** – Plectenchymatous (Type B) to transitional (Type H) (Figure A2.15d) to pseudoparenchymatous with epidermoid cells (Type M).

***Cystidia*** - Clusters of brown, bristle-like setae (Type A) abundant on mantle surface (Figure A2.15e). These are thick-walled at the base. Clusters of globular cells (Type G) common on mantle surface (Figure A2.15f).

## Identity

Resembles species in the Thelephoraceae (Agerer & Wiess, 1989).

Figure A2.15 Thelephoroid 1.
(a) Tips unbranched or simply branched. (b) Mantle surface covered with brown setae. (c) Emanating hyphae (bar = 25 μm). (d) Mantle dissection showing outer mantle in plan view (bar = 10 μm). (e) Setae (bar = 25 μm). (f) Clusters of globular cells on mantle surface (arrows, bar = 25 μm).



# A2.16 Thelephoroid 2 (JMCc37)

## Macroscopic features

***Morphology*** – occur singly or as groups on the primary root or at tips of laterals (Figure A2.16a). Tips straight and somewhat granular at the mantle surface. Many long emanating hyphae (Figure A2.16b). These have a reddish-brown hue. Mycorrhiza appears cigar brown (16) in colour.

## Microscopic features

***Hyphae*** – Common. 5-6 µm diameter. Straight to curved (Figure A2.16c). Hyphae thick-walled, smooth-walled and regular in outline (Figure A2.16d). Branching uncommon, furcate to squarrose. Occasional droplets in hyphal cells. Septa common and clamped. Clamps with septum central and aperture present (Figure A2.16d). Hyphae emerge from swollen basal cells in the mantle surface (Figure A2.16e). Basal cells 8 x 22 µm.

***Mantle*** –

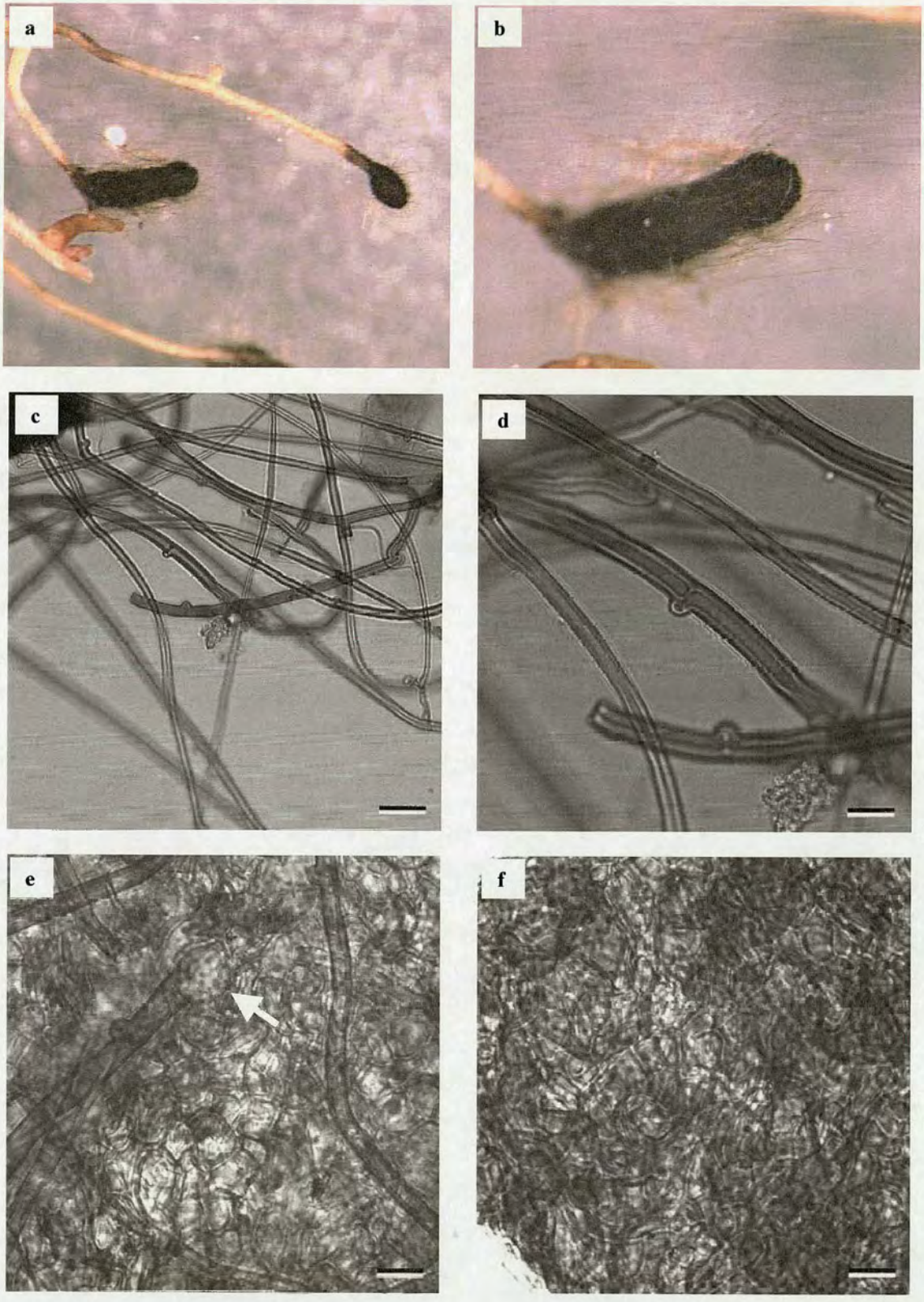
***Outer mantle*** – pseudoparenchyma of large, thick-walled, angular to rounded cells (Figure A2.16e, f).

***Inner mantle*** – plectenchyma of unordered hyphal cells.

## Identity

Closely matches the morphotype "Piceirhiza nigra" described from *Picea abies* (Berg & Gronbach, 1988) which is thought to be formed by a species of *Tomentella*.

Figure A2.16 Thelephoroid 2.
(a) Colonization of distal ends of root laterals. (b) Emanating hyphae long and thick. (c) Emanating hyphae (bar = 25 μm). (d) Clamp connection on emanating hypha (bar = 10 μm). (e) Hyphae emanating from swollen basal cells (arrow) in the mantle (bar = 10 μm). (f) Outer mantle (bar = 10 μm).



# A2.17 *Tricholoma* sp. 1 (JMCc48)

## Macroscopic features

***Morphology*** – Root tips clustered in monopodial pyramidal form. Individual tips straight. Cottony, matte, host not visible.

***Rhizomorphs*** - Abundant white rhizomorphs (Figure A2.17).

## Microscopic features

***Rhizomorphs*** - Very compact. Smooth. Undifferentiated.

***Hyphae*** – Common. 2-3 μm diameter. Tortuous. Appear as tangled mass lying close to mantle edge. Septa common and unclamped. Stain blue in Cotton blue. Hyphal walls smooth. Branching rare.

## Identity

Identified as *Tricholoma* on the basis of its very numerous, very compact, undifferentiated, white rhizomorphs (Agerer, 1987).

Figure A2.17 *Tricholoma* 1.
Mycorrhizas with numerous rhizomorphs.

# A2.18 *Tuber* 1 (JMCc38)

## Macroscopic features

***Morphology*** – Usually single, simple, unbranched root tips (Figure A2.18a) but frequently encountered as clusters with monopodial pinnate branching. Root tips tend to be a greyish colour when young, becoming a pale yellowish-orange. Tips are straight and usually smooth. Occasionally tips are short spiny, usually at their distal ends. The mantle is fully formed, thus obscuring the host cells beneath.

## Microscopic features

***Hyphae*** – Generally rare although occasionally seen in profusion on young tips (Figure A2.18b). 8 - 12 µm at the mantle surface, narrowing to 4 - 5 µm. Hyphae tend to be smooth and somewhat straight at the base, becoming irregular and tortuous away from the mantle edge. Stain darkly in Cotton Blue. Hyphal contents opaque, not hyaline.

***Mantle*** – The mantle surface is characterised by a network of thick (8 - 12 µm) hyphae (Figure A2.18c). Oil droplets appear to be common within the cells of these hyphae.

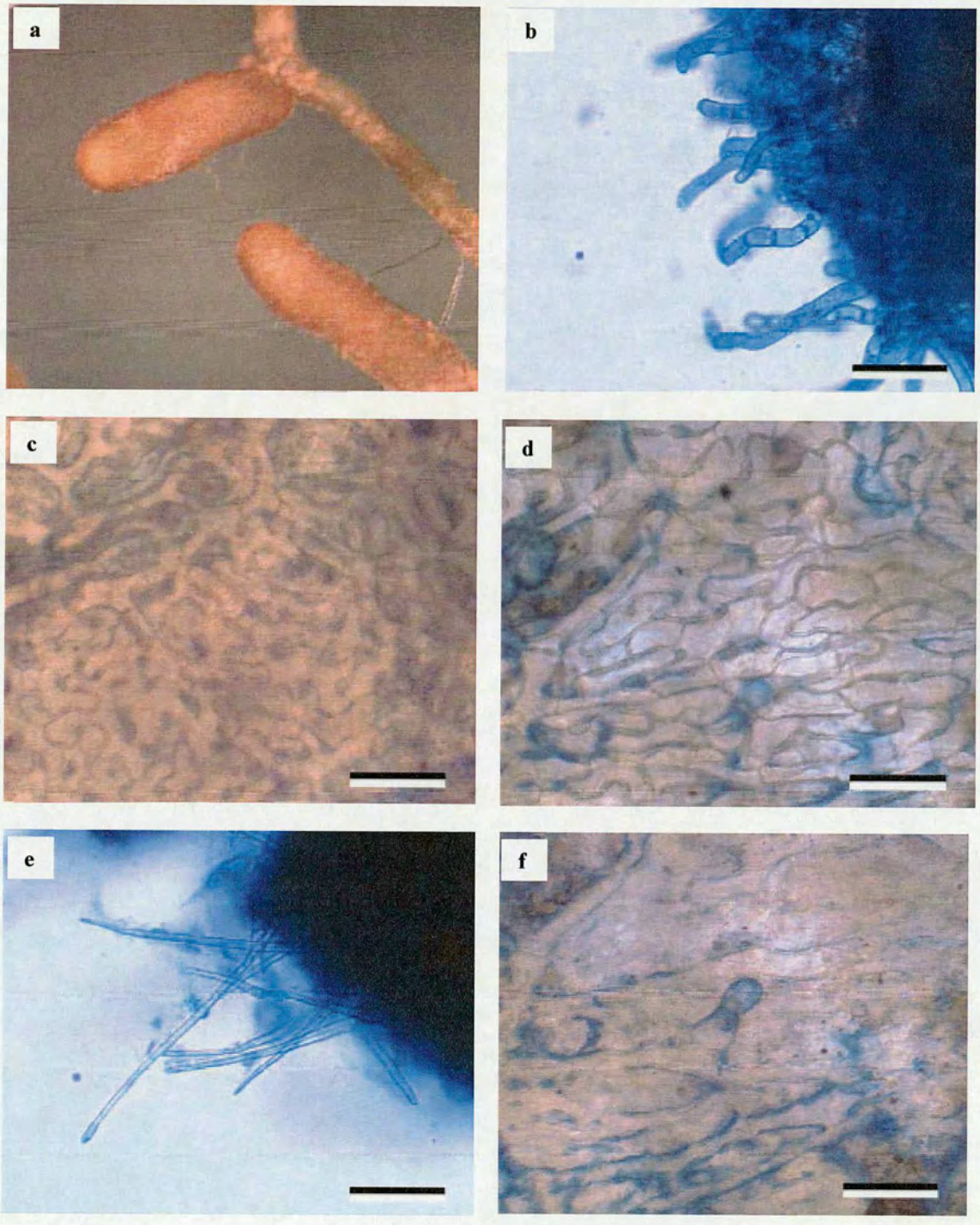
***Outer mantle*** – Pseudoparenchyma of epidermoid cells (Type M). Cells tend to be thick-walled. Patches of elongated cells are occasionally interspersed among the pseudoparenchyma (Figure A2.18d)

***Cystidia*** - Setae are bristle-like (Type A) when present (Figure A2.18e). Globular structures that stain darkly in Cotton Blue (like the emanating hyphae) are commonly observed on the mantle surface (Figure A2.18f). These are smooth and have blunt, rounded tips. They have the appearance of being hyphal tips extending 10 - 20 µm from the mantle surface.

## Identity

Close to *Tuber borchii* (Rauscher *et al.*, 1996). It is separated from *Tuber* 2 (JMCc24) by the presence of a hyphal network on the mantle surface that can be several cells thick, thicker emanating hyphae and the presence of darkly staining globular, cystidia-like structures on the mantle surface. *Tuber 1* is also similar to *Tuber* 3 (JMCc32) but is distinguished from it by the presence of cystidia which are absent from *Tuber* 3.

Figure A2.18 *Tuber* 1.
(a) Tips straight and often appear spiny due to setae. (b) Emanating hyphae (Cotton blue, bar = 25 µm). (c) Hyphal reticulum on mantle surface (bar = 25 µm). (d) Outer mantle (bar = 25 µm). (e) Awl-shaped cystidia (Cotton blue, bar = 25 µm). (f) Globular cystidia (Cotton blue, bar = 25 µm).



# A2.19 *Tuber* 2 (JMCc24)

## Macroscopic features

***Morphology*** – Simple or irregularly branched. Light sandy orange colour. Matte. Tips straight or slightly bent (Figure A2.19a). Finely grainy. Setae frequent on tips and flanks of mycorrhizas (Figure A2.19b). Occasional longer emanating hyphae.

## Microscopic features

***Hyphae*** – 3-4 µm diameter. Rare. Tortuous. Smooth. Unbranched. Septa common. Hyphae stain lightly in cotton blue.

***Mantle*** –

***Outer mantle*** – Outer mantle pseudoparenchymatous with epidermoid cells with thickened gelatinous walls (Agerer Type M) (Figure A2.19c).

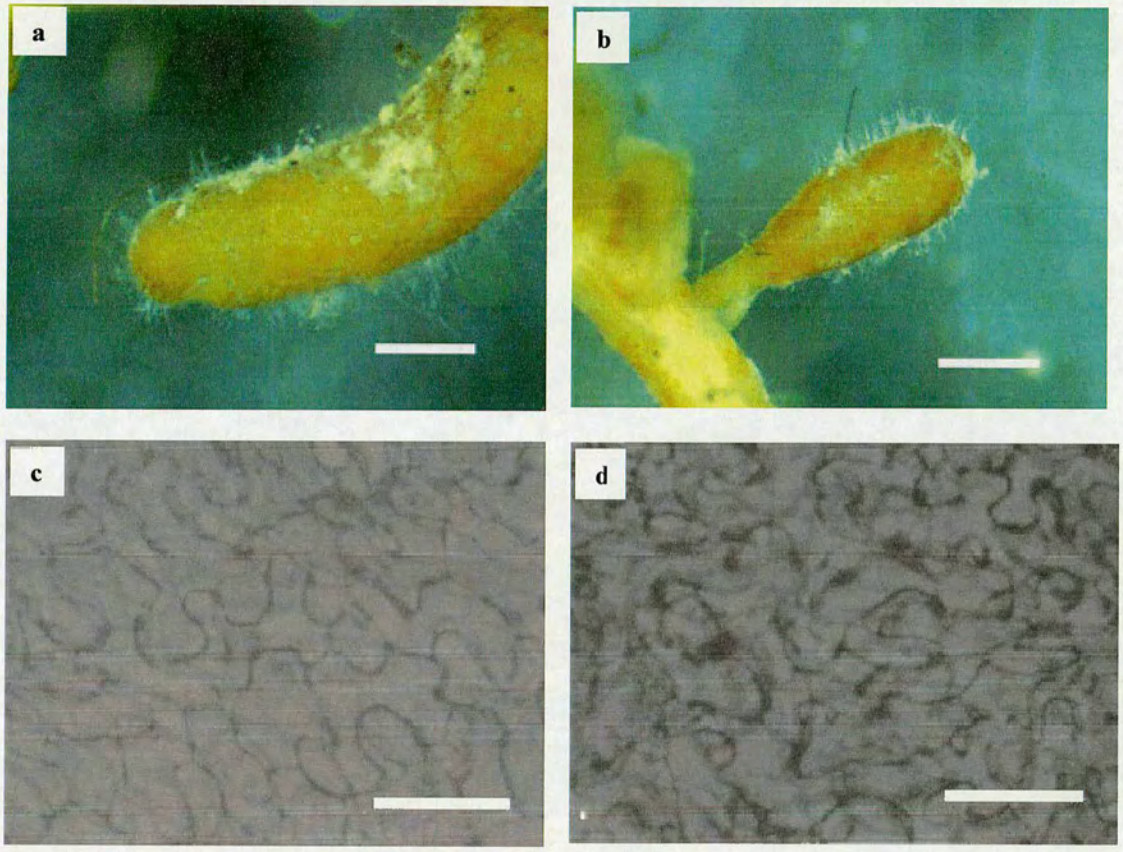
***Inner mantle*** – Pseudoparenchymatous with more sharply defined epidermoid cells (Agerer Type M) (Figure A2.19d).

***Cystidia*** - Frequent setae. Generally 75 (- 105) µm long x 4-6 µm wide at base. Smooth and more or less straight (bristle-like; Agerer Type A). Septa rare. Basal cell usually appears round but occasionally rectangular.

## Identity

Macroscopically very similar to *Tuber* 1 (JMCc38) in being a sandy orange colour and having numerous, awl-shaped setae. They differ from each other in *Tuber* 1 having a well-developed hyphal reticulum overlying the outer mantle. This is not present in *Tuber* 2. *Tuber* 3 (JMCc32) also has a well developed reticulum but differs in having no or very rare setae.

Figure A2.19 *Tuber* 2.
(a) Root tips can appear bent (bar = 1 mm). (b) Setae common on tip and flanks of mycorrhiza (bar = 2 mm). (c) Outer mantle (bar = 10 µm). (d) Inner mantle (bar = 10 µm).



# A2.20 *Tuber* 3 (JMCc32)

## Macroscopic features

***Morphology*** – Tips usually simple (Figure A2.20a, Figure A2.20b) or occasionally irregularly branched (Figure A2.20c). 2.5 - 3.0 mm long x 0.4 - 0.5 mm wide. Light brown in colour, often paler towards distal ends. Smooth. Setae absent or very rare.

## Microscopic features

***Hyphae*** – Rare. 4-6 µm diameter. Tortuous. Originate from hyphal net covering mantle. Hyphae not extending far from the mantle surface. Rarely branched. Where branched, perpendicular, usually with septum at base of branch.

***Mantle*** – Pseudoparenchymatous throughout. Mantle surface covered by coarse network of wide hyphae. These surface hyphae often stain well in cotton blue (Figure A2.20d).

***Outer mantle*** – Pseudoparenchymatous with epidermoid cells with much thickened walls (Figure A2.20e, Figure A2.20f).

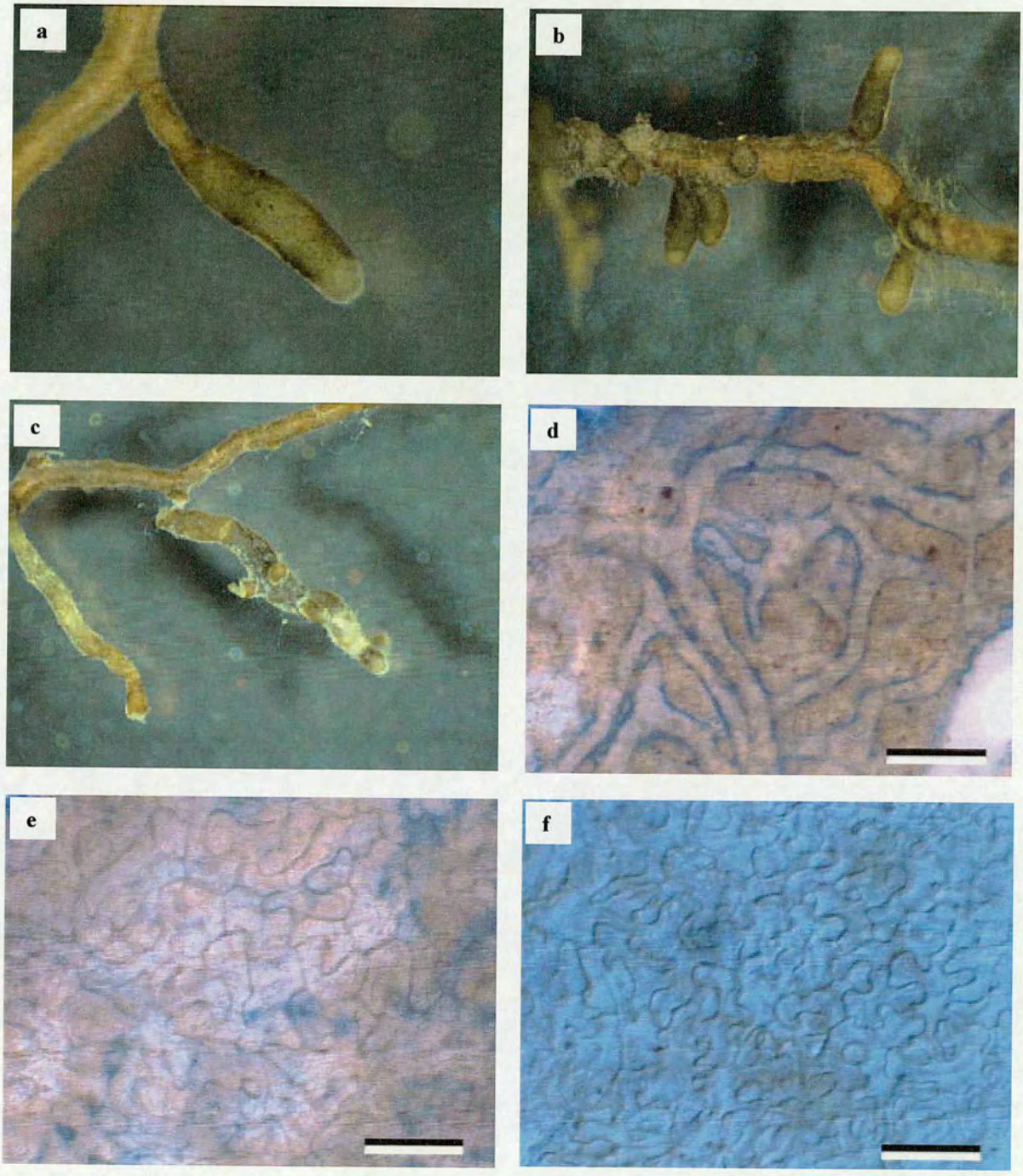
***Inner mantle*** – Pseudoparenchymatous with epidermoid cells. Cell walls not thickened. Cells becoming less sinuous down through mantle layers. Innermost layer includes some elongated cells.

***Cystidia*** - Usually absent. Occasionally present in low numbers. When present, usually bristle-like with septum towards base. Very rarely clavate with several septa.

## Identity

Similar to *Tuber rufum* which is the only *Tuber* morphotype with epidermoid cells that rarely forms cystidia (Rauscher *et al.*, 1995).

Figure A2.20 *Tuber* 3.
(a) Tips simple, smooth, straight. (b) Clusters of simple tips arising from the primary root. (c) Mycorrhizas occasionally irregularly branched. (d) Mantle surface covered by coarse network of hyphae (Cotton blue, bar = 25 µm). (e) Outer mantle a pseudoparenchyma of epidermoid cells (Cotton blue, bar = 25 µm). (f) Outer mantle (DIC, bar = 25 µm).



# A2.21 Unknown 1 (JMCc01)

## Macroscopic features

***Morphology*** – Branching monopodial pyramidal, irregular (Figure A2.21a) or simple (Figure A2.21b). Relatively little alteration of the underlying root. Pale brown to yellowy-orange to pale. Smooth or with very few emanating hyphae.

## Microscopic features

***Hyphae*** – 2 - 4 µm diameter, usually smooth, occasionally slightly verrucose. Tortuous and occasionally elbowed. Septa can be common or rare and indistinct. Thick-walled. Hyphal branching common. Stain blue in cotton blue, pink/purple in toluidine blue.

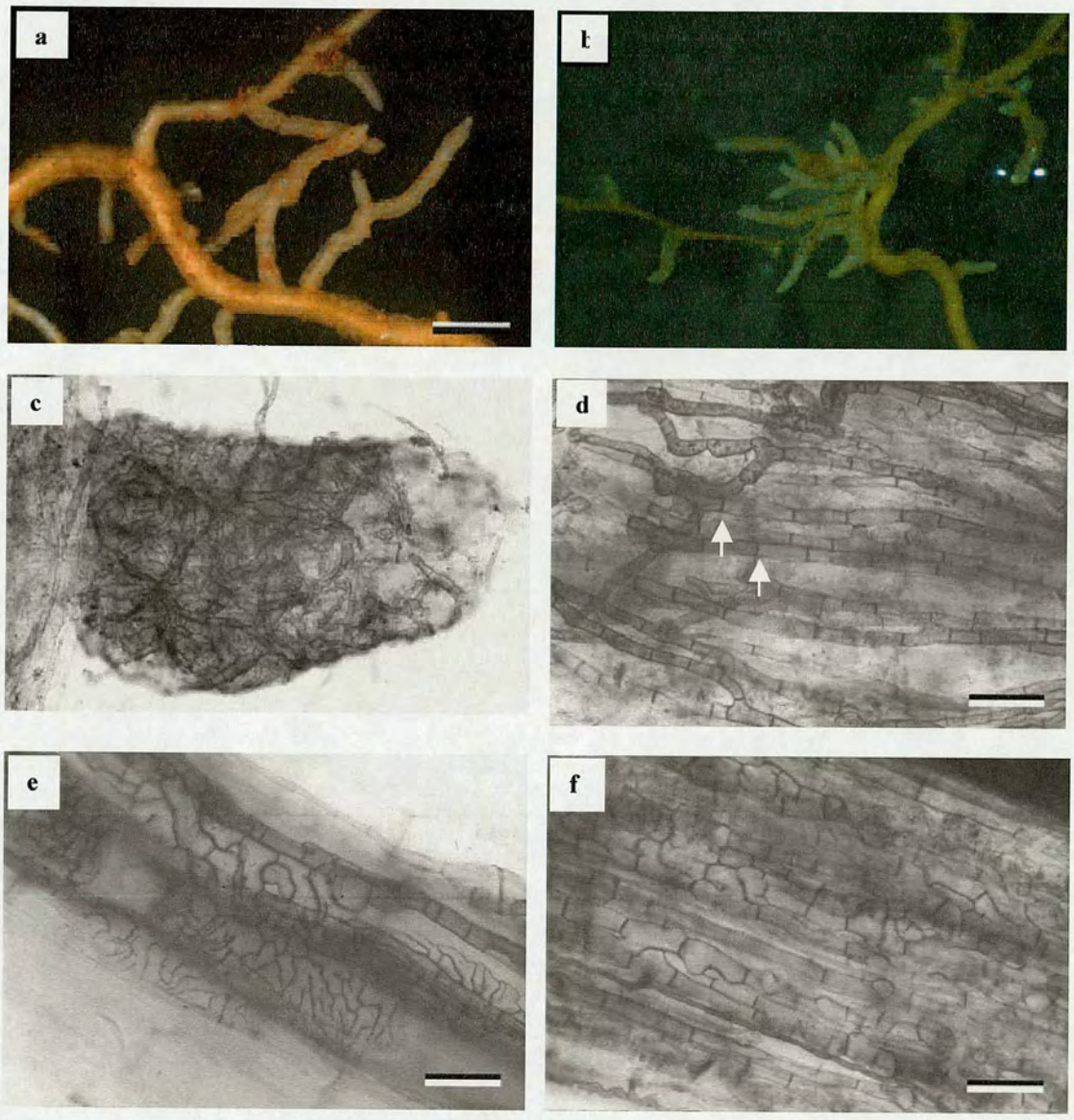
***Mantle*** – Thickened hyphae (6 - 10 µm diameter) on short-root surface forming loose, unorganised pattern (Figure A2.21c).

***Long root colonization*** – This fungus also colonizes extensive portions of the long roots sub-tending the short roots. Long root colonization was characterised by hyphae running along the root surface (Figure A2.21d), or running through the interstitial spaces within the root cortex forming Hartig-net-like structures around some cells (Figure A2.21e). Surface hyphae coalesce in places to form mantle-like patches (Figure A2.21f). Hyphae colonizing the long roots often have characteristic cytoplasmic disjunctions at the septa. In such cases cytoplasm on one side of a septum appears granular and has many inclusions that stain darkly in cotton blue while on the other side it is markedly less granular and without inclusions (Figure A2.21f).

## Identity

Unknown. Affinities of this fungus to species of desert truffles are discussed in Chapter 2.

Figure A2.21 Unknown 1.
(a) Simple branching (bar = 1 mm). (b) Irregular cluster of root tips. (c) Swollen hyphae forming loose, discontinuous mantle on short root. (d) Running hyphae colonizing long root (bar = 25 m) Arrows indicate cytoplasmic disjunctions. (e) Hartig net-like structure in long root (bar = 25 m). (f) Surface hyphae coalescing to form a rudimentary mantle (bar = 25 m).



# A2.22 Unknown 2 (JMCc45)

## Macroscopic features

*Morphology* – Simple unbranched mycorrhizas (Figure A2.22a). Tips straight to bent, cottony. Much soil adhering to emanating hyphae. Host root cells visible through weak mantle.

## Microscopic features

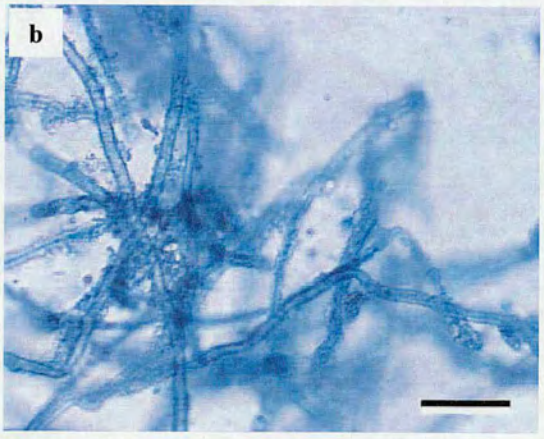
*Hyphae* – Two types
1. Thin (1-2 µm diam.), thick-walled. Septa present but rather rare and only faintly visible.
2. Thicker (2-4 µm diam.), septate (Figure A2.22b).
Both types of hyphae are tortuous to straight, smooth, commonly branched (typically furcate) contain numerous oil bodies and show a variable stain reaction in Cotton blue (blue to no reaction).

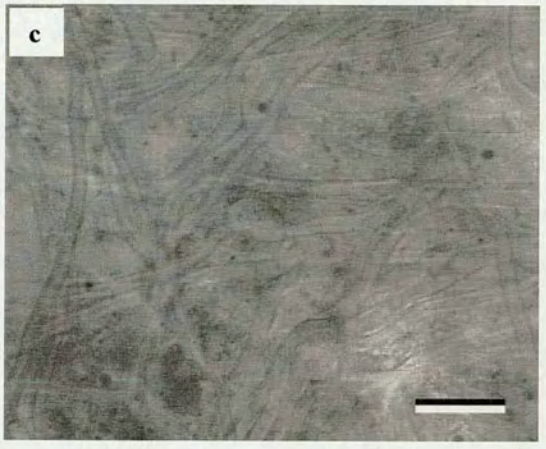
*Mantle* – Generally a loose network of thin hyphae (1.5 - 2.5 µm) (Figure A2.22c). Occasionally more developed with a loose network overlying a plectenchymatous layer. In places the mantle can become consolidated to form two or three layers of unstructured plectenchyma (Agerer Type B).

## Identity

Unknown

Figure A2.22 Unknown 2.
(a) Simple, unbranched mycorrhizas (bar = 1 mm). (b) Emanating hyphae (Cotton blue, bar = 25 μm). (c) Outer mantle (bar = 10 μm).

# A2.23 Unknown 4 (JMCc35)

## Macroscopic features

***Morphology*** – Simple, unbranched, monopodial pinnate (Figure A2.23a) to densely corraloid (Figure A2.23b). When corraloid, usually covered with many adhering soil particles, Tips straight to tortuous, occasionally bent. Colour pale cream to Fulvous (12). Tips can appear translucent when in the paler form. Tips usually cottony with emanating hyphae that are common to abundant.

## Microscopic features

***Hyphae*** – Dimorphic.
Type 1 - 3-4 µm, tortuous, gnarled, often covered with soil particles (Figure A2.23c).
Type 2 - 1-2 µm, straight to curved, smooth-walled. Commonly with Y-shaped branching. Point of branching often slightly enlarged (Figure A2.23d).
Transition from Type 1 to Type 2 is often observable in the same hypha.

***Mantle*** –

***Outer mantle*** – Transitional (Type H). Individual cells small (2-3 µm diameter). Cells commonly embedded in a gelatinous matrix (Figure A2.23e)

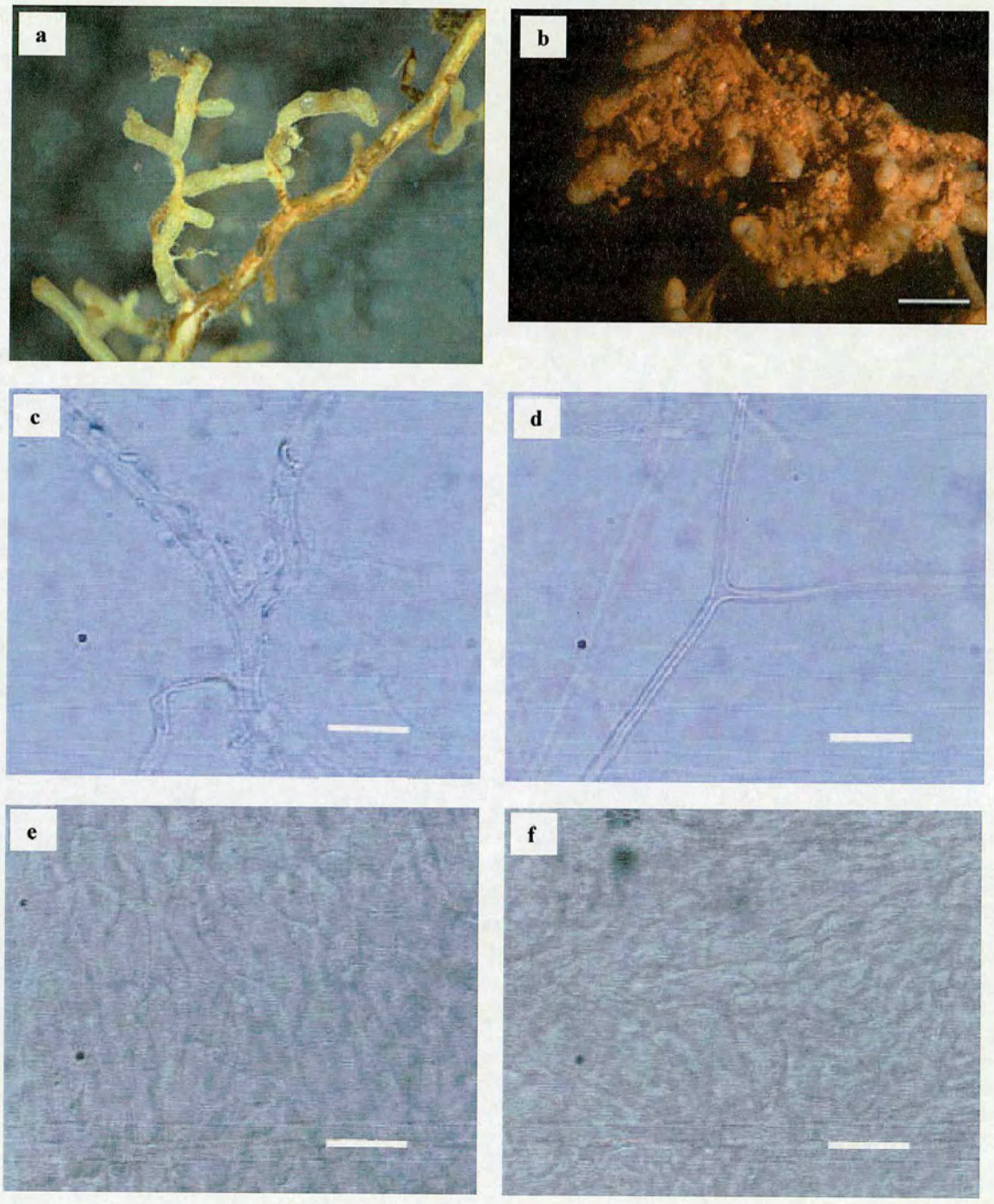
***Middle mantle*** - Plectenchyma of parallel cells (Type B).

***Inner mantle*** – Transitional (Type H). Gelatinous matrix. (Figure A2.23f)

## Identity

Unknown

Figure A2.23 Unknown 4.
(a) Monopodial pinnate branching. (b) Densely corraloid habit. (c) Type 1 hyphae (bar = 10 µm). (d) Type 2 hyphae (bar = 10 µm). (e) Outer mantle (bar = 10 µm). (f) Inner mantle (bar = 10 µm).



# A2.24 Unknown 5 (JMCc44)

## Macroscopic features

***Morphology*** – Occasionally occur as simple unbranched tips (Figure A2.24a, Figure A2.24b) but usually irregularly shaped and irregularly branched mycorrhizal clusters with bent or occasionally straight tips. Abundant long, thin, straight, hyaline hyphae can give the appearance of a 'long spiny' texture. Otherwise cottony. Colour Sepia (26) to Brown Vinaceous (25) to Fulvous (12).

## Microscopic features

***Hyphae*** – Abundant, 1-2 µm diam. Straight to curved or wavy. Septa rare and indistinct and not clamped. Hyphae smooth and unbranched. Variable stain reaction in Cotton Blue (i.e, either stained or not). Hyphae are often dimorphic. The thin hyaline, smooth hyphae are often seen to change to a thicker form that is 2-3 µm diameter, somewhat tortuous and with adhering debris on the surface (Figure A2.24c). The outline of these hyphae is very irregular and knobbly. It is generally these hyphae that stain blue in Cotton Blue with the thinner hyphae remaining unstained.

***Mantle*** –

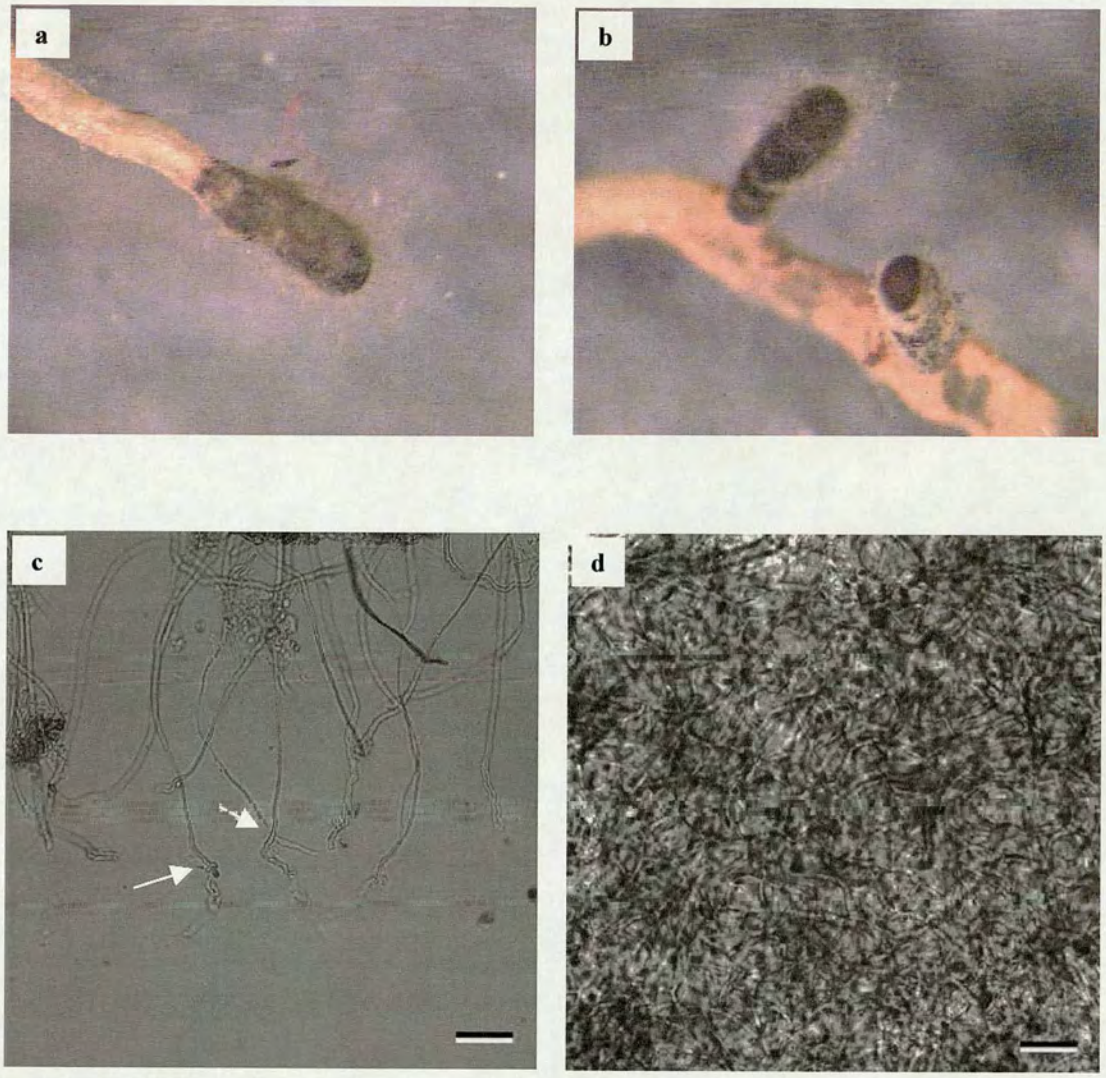
***Outer mantle*** – Pseudoparenchyma of small (4-8 µm), irregular, angular to rounded (mostly angular), thick-walled cells (Figure A2.24d).

***Inner mantle*** – Plectenchyma of thin-walled, densely packed cells.

## Identity

Unknown

Figure A2.24 Unknown 5.
(a) Simple tip arising at end of lateral root. (b) Simple tips arising directly from primary root. (c) Dimorphic emanating hyphae.Arrow point to transition from thin, smooth-walled, uniform hyphal morphology to thicker, irregular morphology (bar = 25 μm). (d) Outer mantle (bar = 10 μm).



## A2.25 Unknown 6 (JMCc42)

### Macroscopic features

***Morphology*** – Simple (Figure A2.25a) or simply branched (Figure A2.25b). Often whole lateral root colonised. Sections of primary root occasionally colonised. Reddish-brown colour. Host cells not generally visible.

### Microscopic features

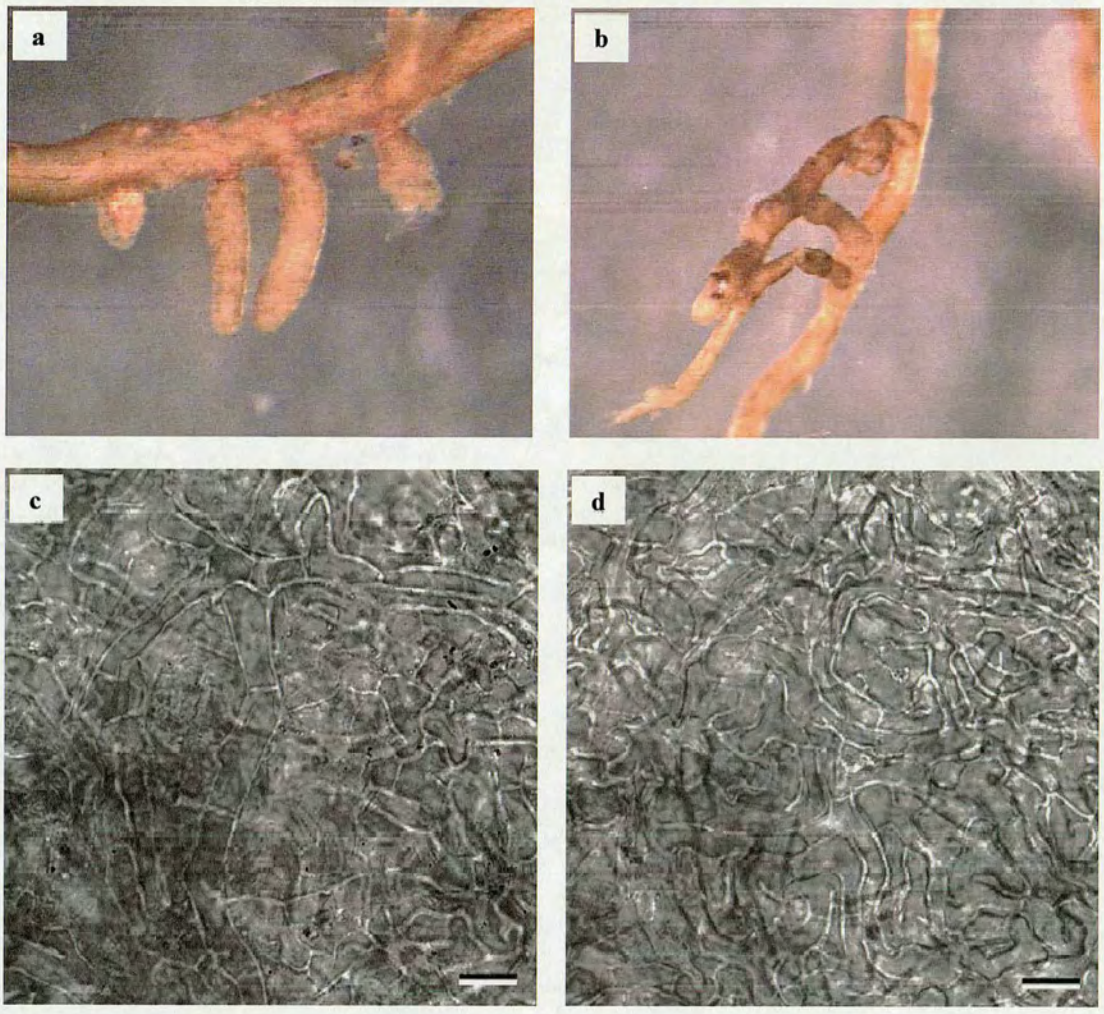
***Hyphae*** – Rare.

***Mantle*** – Mantle surface often with diffuse network of broad hyphae (8-12 μm diameter) (Figure A2.25c). Mantle proper composed of an irregular plectenchyma (Figure A2.25d). Mantle hyphae stain well in Cotton Blue.

### Identity

Unknown.

Figure A2.25 Unknown 6.
(a) Simple tips arising directly from the primary root. (b) Mycorrhiza with simple branching. (c) Hyphal reticulum overlaying outer mantle (bar = 10 μm). (d) Irregular plectenchyma of outer mantle proper (bar = 10 μm).



## A2.26 Unknown 7 (JmCc56)

### Macroscopic features

***Morphology*** – Simple unbranched to monopodial pinnate. Pale brown. Cottony (Figure A2.26a).

### Microscopic features

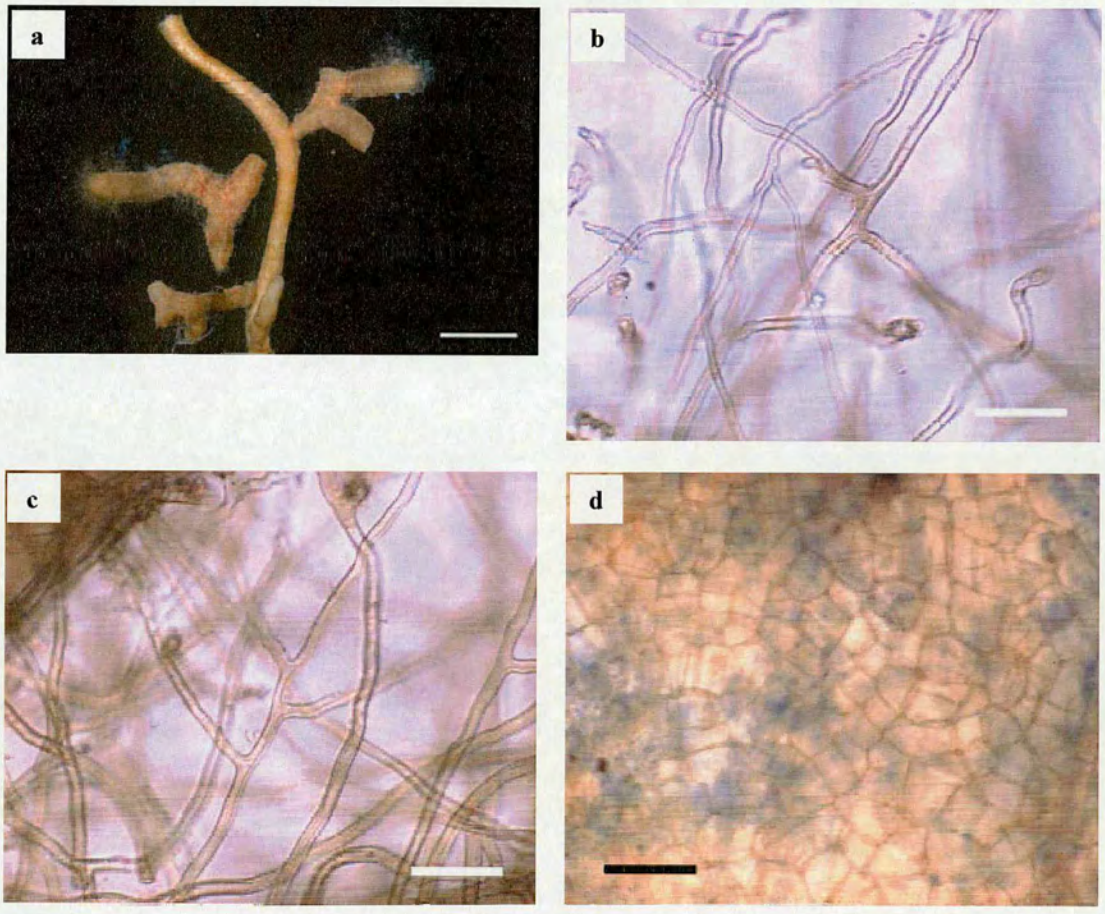
***Hyphae*** – Emanating hyphae abundant. 4 - 6 µm diameter. Light brown. Mostly rather straight, occasionally tortuous in sections. Thick-walled. Smooth to finely verrucose. Reaction to Toluidine blue varying from none to light purple. Septa common, unclamped. Branching common, squarrose (Figure A2.26b) to furcate (Figure A2.26c).

***Mantle*** – Outer mantle strongly pseudoparenchymatous with sharply angular to rounded cells (Figure A2.26d).

### Identity

Unknown.

Figure A2.26 Unknown 7.
(a) Branched mycorrhizas (bar = 1 mm). (b) Hyphae with squarrose branching (bar = 25 μm). (c) Hyphae with furcate branching (bar = 25 μm). (d) Outer mantle (bar = 25 μm).



# A2.27 Unknown 9 (JMCc41)

## Macroscopic features

***Morphology*** – Tips simple or simply branched (Figure A2.27a, Figure A2.27b). Straight, occasionally club-tipped. Greyish in colour. Mantle appears to be thin (host cells visible).

## Microscopic features

***Hyphae*** – Rare. 2-4 µm diameter. Septate. Branching common, often squarrose. Cytoplasm somewhat granular (hyphae opaque). Oil droplets common.

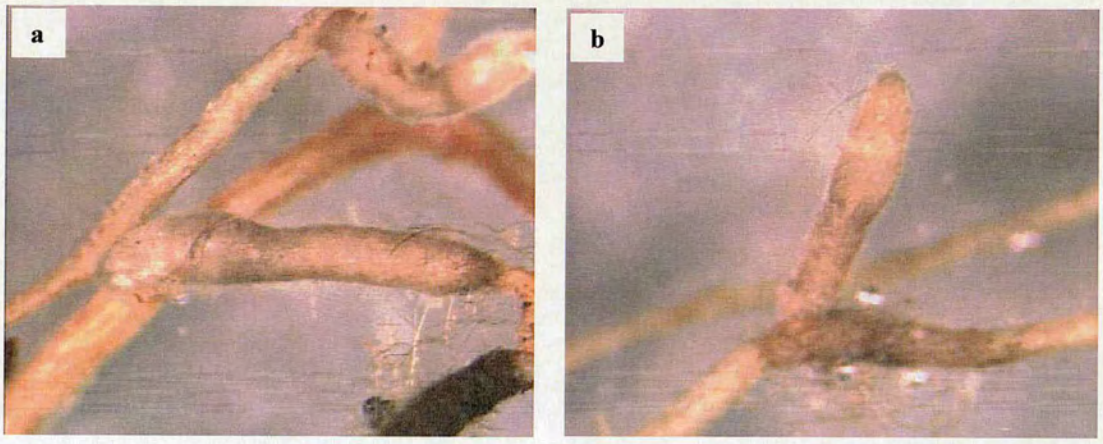
***Mantle*** –

***Outer mantle*** – Irregular plectenchyma (Agerer Type B). Occasionally see patches of pseudoparenchyma of irregular cells at tips.

## Identity

Unknown

Figure A2.27 Unknown 9.
(a), (b) Simple tips



# A2.28 Unknown 11 (JMCc52)

## Macroscopic features

***Morphology*** – Monopodial pinnate (Figure A2.28a) to irregularly branched clusters (Figure A2.28b). Cigar brown (16). Tips straight to bent to tortuous. Thick black hyphae in attendance, usually in a mass at the base of mycorrhiza and on the sub-tending root.

## Microscopic features

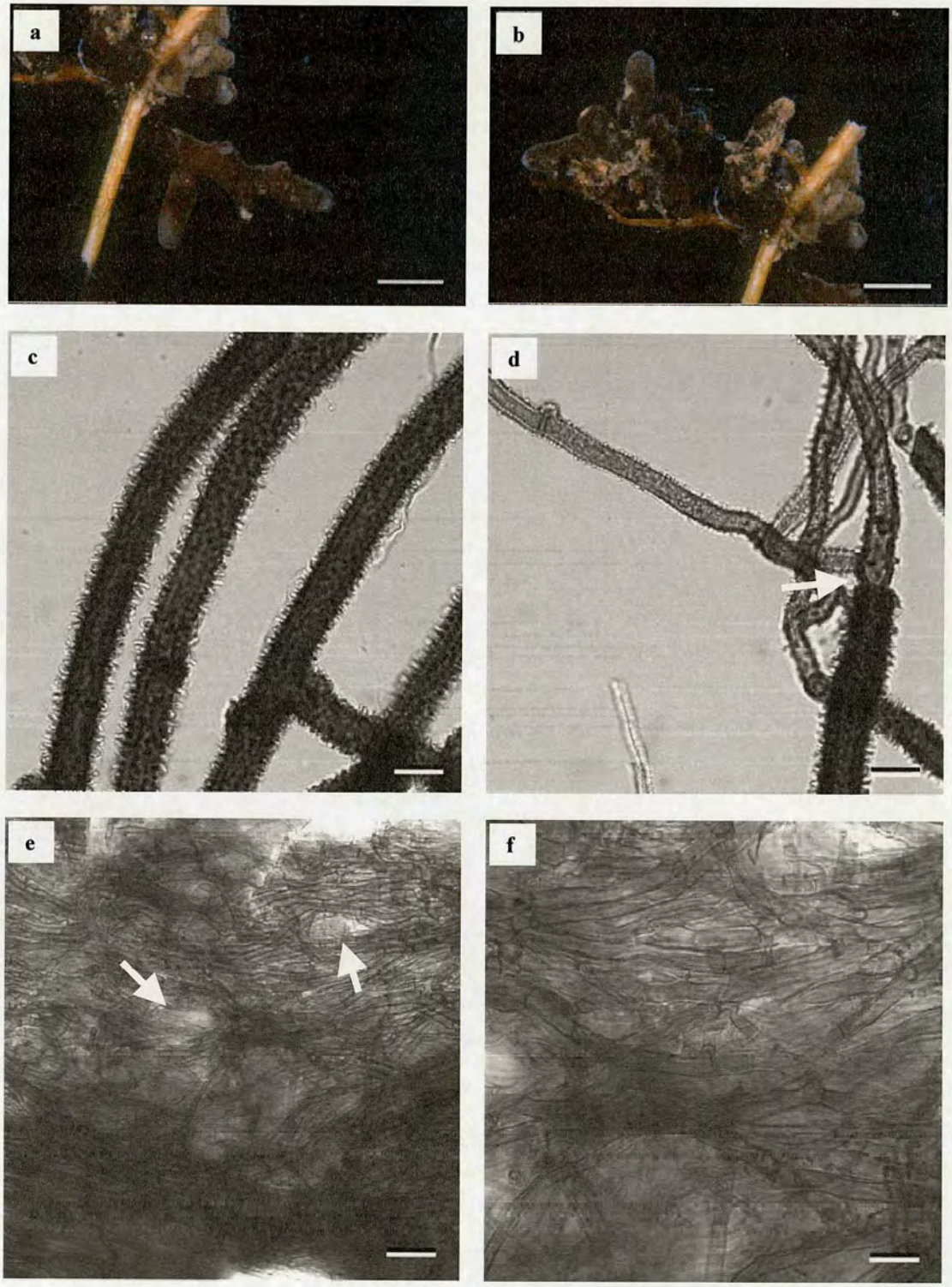
***Hyphae*** – Dark brown. 4 μm at mantle surface, increasing to 10 μm. Smooth close to mantle, becoming richly ornamented with spines (Figure A2.28c). Clamped septa rather rare. Branching rare. Proximal ends often with sharp disjunction between thick old hyphae and thinner hyphae appearing to emerge from these (Figure A2.28d).

***Mantle*** – Plectenchymatous with ring-like arrangement of hyphal bundles (Agerer Type A) (Figure A2.28e). Mantle hyphae unornamented and commonly branched (Figure A2.28f). Hyphae in lower layers forming a more irregular plectenchyma (Agerer Type B).

## Identity

Unknown

Figure A2.28 Unknown 11.
(a) Monopodial branching (bar = 1 mm). (b) Irregularly branched cluster (bar = 1 mm). (c) Emanting hyphae richly ornamented (bar = 10 μm). (d) Hyphal disjunction (arrow, bar = 10 μm). (e) Outer mantle showing ring-like arrangement of hyphae (arrows, bar = 25 μm). (f) Outer mantle hyphae (bar = 10 μm).



## A2.29 Unknown 12 (JMCc53)

### Macroscopic features

***Morphology*** – Simple, unbranched to monopodial pinnate branching (Figure A2.29a) to irregular clusters (Figure A2.29b). Dark brick (20) to Bay (19) to Cigar brown (16). Tips straight, smooth and somewhat shiny.

### Microscopic features

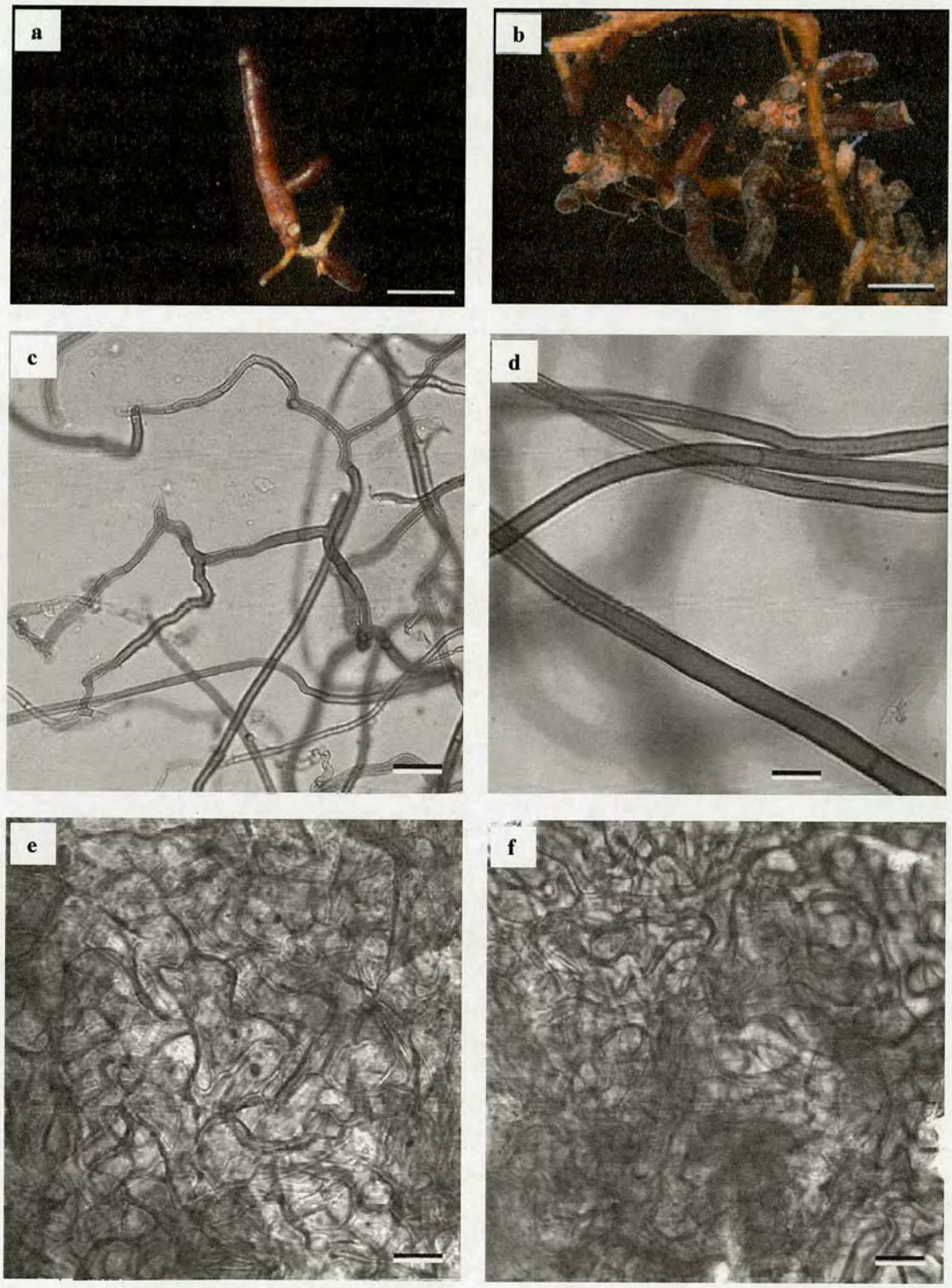
***Hyphae*** – Emanating hyphae rare. 4 – 6 μm diameter. Brown. Branching rare, squarrose when occurring (Figure A2.29c). Straight, thick-walled, smooth-walled (Figure A2.29d). Septa common and unclamped.

***Mantle*** – Outer mantle transitional between pseudoparenchymatous and plectenchymatous (Agerer Type H) (Figure A2.29e). Inner mantle plectenchymatous (Figure A2.29f).

### Identity

Unknown.

Figure A2.29 Unknown 12.
(a) Monopodial pinnate branching (bar = 1mm) (b) Cluster of mycorrhizal tips (bar = 1mm). (c) Branched hyphae (bar = 25 μm). (d) Hyphae (bar = 10 μm). (e) Outer mantle (bar = 10 μm). (f) Inner mantle (bar = 10 μm).



# A2.30 Unknown 14 (JMCc40)

## Macroscopic features

***Morphology*** – Clusters of irregularly branched tortuous to bent tips (Figure A2.30a). Felty texture. Colour Date Brown (24).

***Rhizomorphs*** - Common, compact, pale (Figure A2.30b).

## Microscopic features

***Rhizomorphs*** - Composed of several compact strands composed of many thin (< 1 µm) hyphae (Figure A2.30c). Strands rather loosely woven together to form a single rhizomorph. Single strands often seen occurring individually. Some swollen cells on surface. Restricted point attachment to mycorrhizas.

***Hyphae*** – Very thin (< 1 µm). Tortuous, septate, hyaline (Figure A2.30d). Frequently appear to have swollen tips.

***Mantle*** –

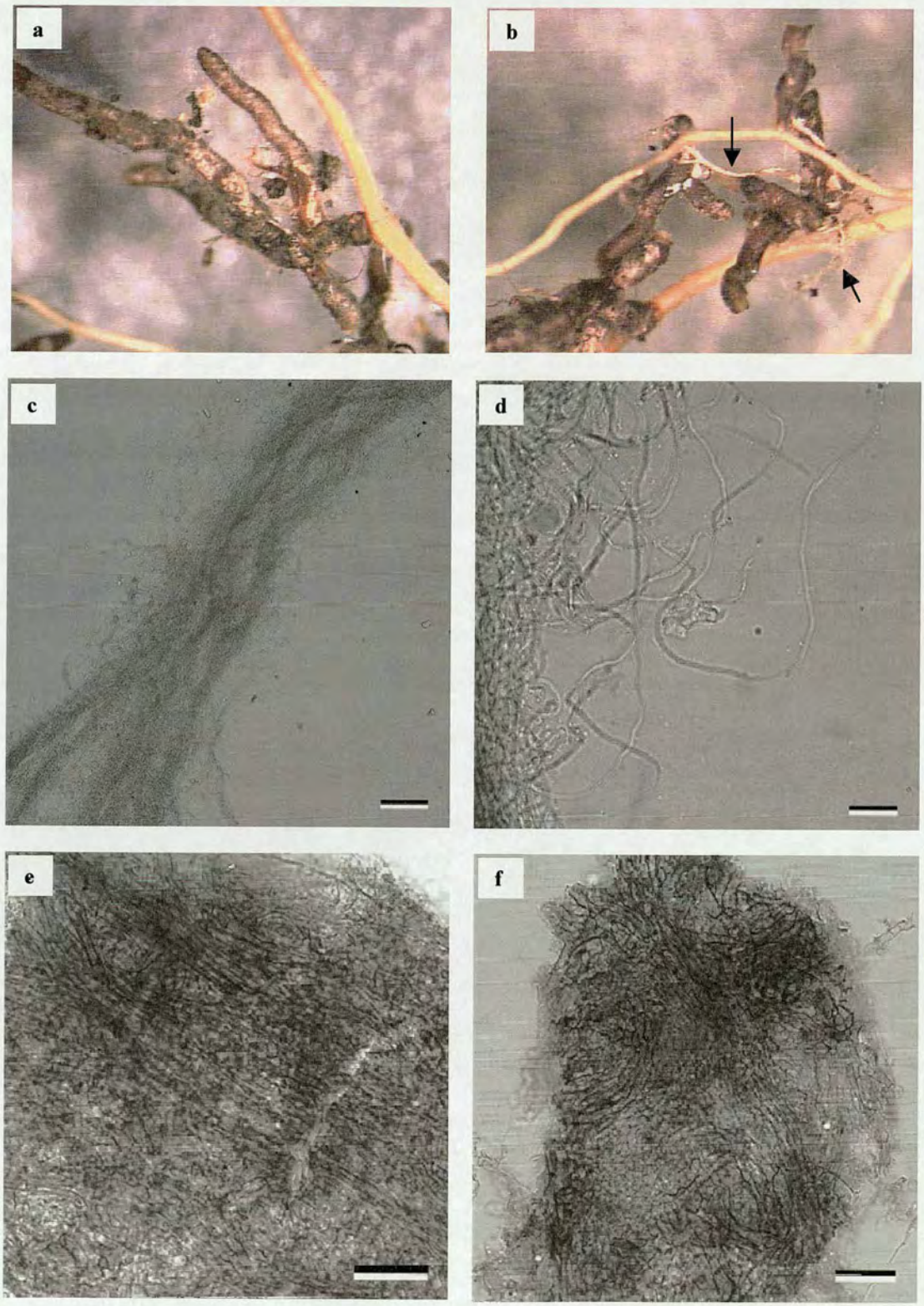
***Outer mantle*** – Plectenchyma (Figure A2.30e). Hyphae 2-3 µm diameter. Hyphal net embedded in gelatinous matrix.

***Inner mantle*** – Transitional to pseudoparenchymatous (Figure A2.30f). Hyphal cells 2-4 µm diameter. Gelatinous matrix.

## Identity

Hyphae and rhizomorphs similar to *Geastrum fimbriata* (Agerer & Beenken, 1998) but JMCc40 has none of the cystidia seen in that mycorrhiza.

Figure A2.30 Unknown 14.
(a) Cluster of irregular, tortuous root tips (b) Rhizomorphs (arrows). (c) Rhizomorph (bar = 25 μm). (d) Emanating hyphae (bar = 10 μm). (e) Outer mantle (bar = 25 μm). (f) Inner mantle (bar = 25 μm).



**Appendix 3.** Values and standard errors for the vertical distribution of fungi within spatial groups 1-6 depicted in Figure 3.6 in Chapter 3. X, Y co-ordinates correspond to cells in the spatial grid within which morphotypes were recorded (see figure3.2 in Chapter 3). X co-ordinates correspond to the distance from the primary root (cm) and Y co-ordinates correspond to root depth (cm). EM = total number of colonized root tips, Group = number of root tips colonized by fungi in each spatial group.

| | x | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | y | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 4 | 5 | 5 | 5 | 5 | 5 | 5 | 6 | 6 | 6 | 6 | 6 | 6 | 7 | 7 | 7 | 7 | 7 | 7 |
| Group 1 | EM | 8.3 | 1.3 | 0.2 | 0.1 | 0.1 | 0.0 | 6.7 | 0.4 | 0.3 | 0.2 | 0.1 | 0.0 | 5.8 | 0.2 | 0.1 | 0.0 | 0.0 | 0.0 | 5.7 | 0.1 | 0.3 | 0.0 | 0.0 | 0.0 | 2.7 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 2.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | n | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 63 | 63 | 63 | 63 | 63 | 63 | 56 | 56 | 56 | 56 | 56 | 56 | 44 | 44 | 44 | 44 | 44 | 44 | 32 | 32 | 32 | 32 | 32 | 32 |
| | se | 1.2 | 0.5 | 0.1 | 0.2 | 0.0 | 0.0 | 0.8 | 0.3 | 0.2 | 0.1 | 0.1 | 0.0 | 0.9 | 0.2 | 0.1 | 0.0 | 0.0 | 0.0 | 1.1 | 0.1 | 0.3 | 0.0 | 0.0 | 0.0 | 0.6 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | Group 1 | 7.6 | 1.3 | 0.2 | 0.0 | 0.0 | 0.0 | 6.0 | 0.4 | 0.3 | 0.2 | 0.1 | 0.0 | 3.3 | 0.2 | 0.1 | 0.0 | 0.0 | 0.0 | 1.8 | 0.1 | 0.3 | 0.0 | 0.0 | 0.0 | 0.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | n | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 65 | 63 | 63 | 63 | 63 | 63 | 63 | 56 | 56 | 56 | 56 | 56 | 56 | 44 | 44 | 44 | 44 | 44 | 44 | 32 | 32 | 32 | 32 | 32 | 32 |
| | se | 0.9 | 0.3 | 0.1 | 0.0 | 0.0 | 0.0 | 0.9 | 0.1 | 0.2 | 0.1 | 0.1 | 0.0 | 0.4 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.4 | 0.1 | 0.2 | 0.0 | 0.0 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Group 2 | EM | 9.1 | 1.1 | 0.2 | 0.0 | 0.0 | 0.0 | 9.9 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 10.6 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 9.9 | 0.3 | 1.2 | 0.1 | 0.0 | 0.0 | 5.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.4 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 |
| | n | 18.0 | 18.0 | 18.0 | 18.0 | 18.0 | 18.0 | 18.0 | 18.0 | 18.0 | 18.0 | 18.0 | 18.0 | 18.0 | 18.0 | 18.0 | 18.0 | 18.0 | 18.0 | 17.0 | 17.0 | 17.0 | 17.0 | 17.0 | 17.0 | 16.0 | 16.0 | 16.0 | 16.0 | 16.0 | 16.0 | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 | 11.0 | 11.0 | 11.0 | 11.0 | 11.0 | 11.0 |
| | se | 2.2 | 0.6 | 0.1 | 0.0 | 0.0 | 0.0 | 3.4 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 2.1 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 2.9 | 0.3 | 0.9 | 0.1 | 0.0 | 0.0 | 1.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 |
| | Group 2 | 1.6 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 4.6 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 6.6 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 6.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | n | 19.0 | 19.0 | 19.0 | 19.0 | 19.0 | 19.0 | 19.0 | 19.0 | 19.0 | 19.0 | 19.0 | 19.0 | 19.0 | 19.0 | 19.0 | 19.0 | 19.0 | 19.0 | 18.0 | 18.0 | 18.0 | 18.0 | 18.0 | 18.0 | 17.0 | 17.0 | 17.0 | 17.0 | 17.0 | 17.0 | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 | 11.0 | 11.0 | 11.0 | 11.0 | 11.0 | 11.0 |
| | se | 1.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 2.4 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 2.2 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 2.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Group 3 | EM | 7.0 | 0.9 | 0.3 | 0.2 | 0.1 | 0.0 | 5.1 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 5.2 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 5.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 4.3 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 2.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 |
| | n | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 28.0 | 28.0 | 28.0 | 28.0 | 28.0 | 28.0 | 28.0 | 28.0 | 28.0 | 28.0 | 28.0 | 28.0 | 26.0 | 26.0 | 26.0 | 26.0 | 26.0 | 26.0 | 20.0 | 20.0 | 20.0 | 20.0 | 20.0 | 20.0 |
| | se | 1.3 | 0.4 | 0.2 | 0.2 | 0.1 | 0.0 | 1.0 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 1.2 | 0.1 | 0.1 | 0.0 | 0.0 | 0.0 | 1.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.7 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 |
| | Group 3 | 0.2 | 0.0 | 0.0 | 0.2 | 0.0 | 0.0 | 0.4 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 2.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 3.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.6 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 2.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | n | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 | 30.0 |
| | se | 0.2 | 0.0 | 0.0 | 0.2 | 0.0 | 0.0 | 0.3 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.7 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Group 4 | EM | 9.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 7.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 5.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 13.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | n | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| | se | 4.7 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 3.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 6.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | Group 4 | 2.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 6.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | n | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 | 3.0 |
| | se | 2.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Group 5 | EM | 19.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 13.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 4.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 5.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | n | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| | se | 11.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | Group 5 | 12.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 5.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | n | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| | se | 12.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Group 6 | EM | 12.0 | 3.3 | 0.7 | 0.7 | 0.2 | 0.0 | 5.6 | 1.3 | 1.0 | 0.3 | 0.5 | 0.2 | 8.0 | 1.0 | 0.4 | 0.0 | 0.1 | 0.0 | 8.7 | 0.5 | 0.6 | 0.0 | 0.0 | 0.0 | 6.3 | -0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 2.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | n | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 9.0 | 9.0 | 9.0 | 9.0 | 9.0 | 9.0 | 9.0 | 9.0 | 9.0 | 9.0 | 9.0 | 9.0 | 6.0 | 6.0 | 6.0 | 6.0 | 6.0 | 6.0 |
| | se | 3.9 | 1.5 | 0.6 | 0.7 | 0.2 | 0.0 | 2.1 | 1.3 | 0.8 | 0.3 | 0.5 | 0.1 | 3.9 | 0.9 | 0.5 | 0.0 | 0.1 | 0.0 | 4.5 | 0.5 | 0.6 | 0.0 | 0.0 | 0.0 | 3.2 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 1.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | Group 6 | 0.0 | 0.0 | 0.1 | 0.1 | 0.0 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 4.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 3.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 2.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | n | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 |
| | se | 0.0 | 0.0 | 0.1 | 0.1 | 0.0 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |